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# **Role of the protein Sus1 and its interaction with the Sac3<sup>CID</sup> motif in transcription-coupled mRNA export**

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                     Dr. Oliver Gruss**

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## **DECLARATION**

I hereby declare that I have written the submitted dissertation myself and in this process have used no other sources or materials than those expressly indicated.

December 17, 2009

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(Christoph Klöckner)

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## SUMMARY

The process of gene expression in the nucleus consists of gene transcription into mRNA, posttranscriptional modification of transcripts and finally export of mRNA through the nuclear pore complex into the cytoplasm before translation into proteins can occur.

Two of the protein complexes proposed to be involved in coupling of transcription and mRNA export are the SAGA complex (Spt-Ada-Gcn5-acetyltransferase) mediating transcription activation through histone acetylation and deubiquitination, and the TREX-2 (Sac3-Thp1-Cdc31-Sus1) complex, which interacts with the nuclear pore complex and is involved in the export of mRNA.

Sac3, which is a central subunit of TREX-2, is suggested to integrate transcription and mRNA export through the CID (Cdc31-interacting domain) motif, which comprises ~80 amino acids in the C-terminal part of the protein. This motif recruits Cdc31, a calmodulin-like protein, and Sus1, a protein functioning in transcription as well as mRNA export. In addition, the CID motif is required for correct targeting of TREX-2 to nuclear pore complexes.

The observation that Sac3<sup>CID</sup> acts as a crucial binding platform in TREX-2 and is also responsible for the interaction with nuclear pores makes it an attractive target to further unravel the genetic network of factors involved in transcription-coupled mRNA export. Mutants from a synthetic lethal (SL) screen with a *sac3Δ* allele have been analyzed for a genetic interaction with *SAC3<sup>CID</sup>* to identify factors that are specifically linked to transcription-coupled mRNA export.

Among the SL strains that proved to be genetically interacting with *SAC3<sup>CID</sup>*, one strain was complemented by *BUR6*, the gene for a transcriptional regulator. Another strain could be complemented by *SPE3*, encoding the spermidine synthase. The small and basic molecule spermidine, produced by Spe3, was suggested to be involved in various nuclear processes like transcriptional repression and global chromatin organisation by interaction of spermidine with chromatin. These novel findings strengthen the connection of Sac3<sup>CID</sup> with transcriptional regulation.

In addition to the search for novel genetic interactions, a complementary biochemical approach to study the link between transcription and export of mRNA was applied. Through a mutational analysis of Sus1, a factor interacting with Sac3<sup>CID</sup> inside

TREX-2 as well as with the transcriptional coactivator SAGA, it was possible to identify conserved residues that are crucial for the interaction of Sus1 with its partner proteins. One of the engineered *sus1* alleles showed significant loss of interaction with both SAGA and TREX-2. In contrast, two other alleles yielded highly reduced interaction with TREX-2 while interaction with SAGA remained undisturbed. Biochemical, cell biological and genetic experiments with these alleles substantiated that the binding of Sus1 to Sac3<sup>CID</sup> (and therefore TREX-2) was disrupted without affecting the interaction with the SAGA complex. A mutational analysis of the single amino acid mutations revealed their contribution to the defects of the combined alleles. These findings will help to define the selectivity and specificity of how Sus1 interacts with the complexes SAGA and TREX-2.

Taken together, the results of my PhD thesis have revealed novel interactions for the genetic network of the pivotal motif *SAC3*<sup>CID</sup> while the creation of alleles that selectively uncouple Sus1 from TREX-2 provides the basis for a detailed analysis of the functions of the versatile protein Sus1 in transcription and mRNA export.



## ZUSAMMENFASSUNG

Der Prozess der Genexpression im Zellkern besteht aus der Transkription eines Genes in mRNA, posttranskriptionellen Modifikationen der Transkripte und schließlich dem Export der mRNA durch den nukleären Porenkomplex ins Cytoplasma, bevor die Proteintranslation ablaufen kann.

Zwei der Proteinkomplexe, die vermutlich an der Kopplung zwischen Transkription und Export von mRNA beteiligt sind, sind der SAGA-Komplex (Spt-Ada-Gcn5-Acetyltransferase), der die Transkriptionsaktivierung durch Acetylierung und Deubiquitinierung von Histonen vermittelt, und der TREX-2- oder auch Sac3-Thp1-Cdc31-Sus1-Komplex, der mit der nukleären Pore interagiert und am Export von mRNA beteiligt ist.

Es scheint, dass Sac3, eine zentrale Untereinheit von TREX-2, die Transkriptionsaktivierung und den Export von mRNA mittels des CID-Motivs (Cdc31-Interaktionsdomäne), welches ~80 Aminosäuren im C-terminalen Anteil des Proteins umfasst, integriert. Dieses Motiv rekrutiert Cdc31, ein dem Calmodulin ähnliches Protein, und Sus1, ein Protein mit Funktionen in der Transkription als auch im Export von mRNA. Außerdem wird das CID-Motiv für die korrekte Zielsteuerung von TREX-2 zu nukleären Porenkomplexen benötigt.

Die Beobachtung, dass Sac3<sup>CID</sup> eine äußerst wichtige Bindungsfläche in TREX-2 darstellt und zusätzlich verantwortlich ist für die Interaktion mit der nukleären Pore, macht es zu einem attraktiven Ziel, um das genetische Netzwerk von Faktoren, die im Transkriptions-gekoppelten Export von mRNA involviert sind, noch weiter aufzuklären. Mutanten aus einem „synthetic lethal screen“ (SL screen) mit dem *sac3Δ*-Allel wurden im Hinblick auf eine genetische Interaktion mit *SAC3*<sup>CID</sup> untersucht, um weitere Faktoren zu identifizieren, die in einer genetischen Beziehung mit Transkriptions-gekoppeltem mRNA-Export stehen.

Von den SL-Stämmen, bei denen eine genetische Beziehung mit *SAC3*<sup>CID</sup> gezeigt werden konnte, wurde ein Stamm durch *BUR6*, dem Gen für einen transkriptionellen Regulator, komplementiert. Ein anderer Stamm konnte durch *SPE3*, dem Gen für Spermidinsynthase, komplementiert werden. Das kleine, basische Molekül Spermidin, das von Spe3 produziert wird, wird mit verschiedenen nukleären Prozessen wie transkriptioneller Repression und globaler Organisation von Chromatin durch

Interaktion mit Spermidin in Zusammenhang gebracht. Diese neuen Ergebnisse stärken die Verbindung von *SAC3<sup>CID</sup>* mit transkriptionellen Mechanismen.

Zusätzlich zu der Suche nach neuen genetischen Interaktionen wurde ein ergänzender Ansatz verfolgt, um die Verbindung zwischen Transkription und Export von mRNA zu untersuchen. Mittels einer Mutationsanalyse von *Sus1*, einem Faktor, der mit *Sac3<sup>CID</sup>* innerhalb TREX-2 als auch mit dem Transkriptions-Koaktivator SAGA interagiert, war es möglich, konservierte Aminosäuren zu identifizieren, die wichtig für die Interaktion von *Sus1* mit seinen Partnerproteinen sind. Eins der erzeugten Allele von *sus1* wies einen signifikanten Verlust der Interaktion mit sowohl SAGA als auch TREX-2 auf. Im Gegensatz dazu ergaben zwei andere Allele eine stark reduzierte Interaktion mit TREX-2, während die Interaktion mit SAGA ungestört blieb. Biochemische, zellbiologische und genetische Experimente mit diesen Allelen erhärteten die Vermutung, dass die Bindung von *Sus1* an *Sac3<sup>CID</sup>* (und dadurch an TREX-2) gestört wurde ohne die Interaktion mit dem SAGA-Komplex zu beeinflussen. Eine Mutationsanalyse der einzelnen konservierten Aminosäuren machten deren Beitrag zu den Alleldefekten deutlich. Diese Ergebnisse werden dabei helfen, die Selektivität und Spezifität der Interaktionen von *Sus1* mit den Komplexen SAGA und TREX-2 zu definieren.

Zusammenfassend haben die Ergebnisse meiner Dissertation neue Interaktionen im genetischen Netzwerk des zentralen Motivs *SAC3<sup>CID</sup>* offenbart, während die Erzeugung von Allelen, die *Sus1* selektiv vom TREX-2-Komplex entkoppeln, die Basis für eine selektive Analyse der Funktionen des vielseitigen Proteins *Sus1* in der Transkription und dem Export von mRNA zur Verfügung stellt.

# 1. INTRODUCTION

A significant step in the evolution from prokaryotes to eukaryotes is the appearance of intracellular organelles confined by membranes. The introduction of membrane-confined organelles entails a spatial segregation of intracellular processes that previously interlocked in the single prokaryotic cellular interior. The separation of processes by a membrane barrier allows for and requires a more fine-tuned regulation of sequential processes. Through compartmentalization, eukaryotic cells could afford a complex and fine-tuned interplay between separate processes that would be impossible to achieve without confined territories inside the cell.

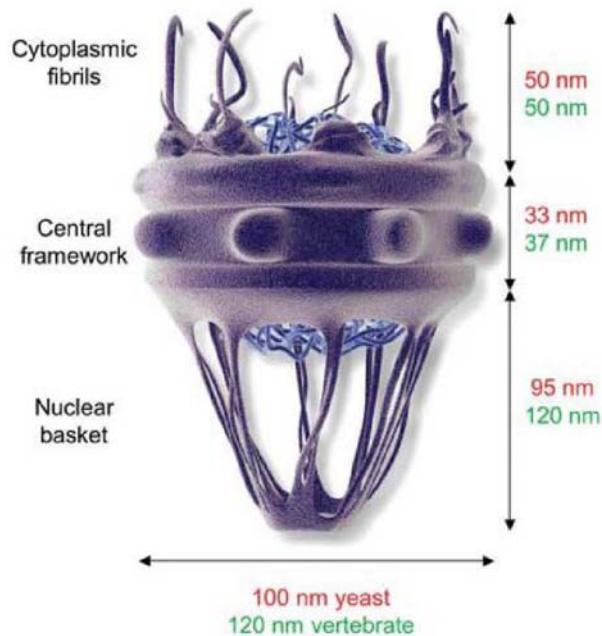
The most dramatic consequence of compartmentalization for eukaryotes concerns the process of gene expression. Genomic DNA is surrounded by the nuclear envelope, a double lipid bilayer, leading to a spatial and functional separation of transcription and translation. This situation makes an effective and selective transport across the nuclear membrane vitally important for the cell.

Nuclear import and nuclear export happen through huge protein complexes spanning the nuclear membrane called nuclear pore complexes (NPCs). NPCs are the only way in and out of the nucleus for trafficking molecules as well as a site of extensive regulation and coupling of different cellular processes.

## 1.1 The nuclear pore complex

Roughly 200 nuclear pore complexes are embedded in the nuclear membrane of a typical yeast cell (Lim et al., 2008) and guarantee the perpetuation of transport between the nucleoplasm and the cytoplasm. These general mediators of nucleocytoplasmic transport show an eightfold rotational symmetry around the central cavity through which the transport processes across the membrane take place (Suntharalingam and Wente, 2003). The central framework of the NPC contains eight spokes which are clamped between a nuclear ring and a cytoplasmic ring on the respective sides of the membrane. Pointing out from the membrane-encircled nuclear pore complex is the so-called nuclear basket on the nuclear side of the membrane and the cytoplasmic fibrils reaching into the cytoplasm. Both of these terminal structures

serve to interact with proteins involved in nuclear transport (D'Angelo and Hetzer, 2008; Fahrenkrog et al., 2001).



**Figure 1 Schematic of the nuclear pore complex (NPC)**

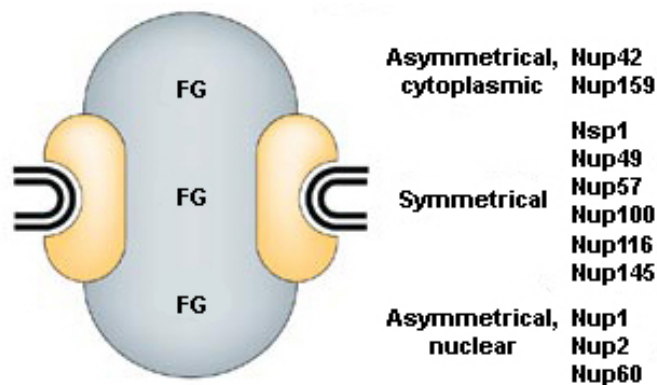
The figure shows a rough representation of the regions of a nuclear pore with the respective sizes given for *S. cerevisiae* (green) and a metazoan (*X. laevis*; red).

Adapted from: <http://sspatel.googlepages.com/nuclearporecomplex>

Nuclear pore complexes in yeast have a molecular weight of ~60 MDa while vertebrate NPCs come up to ~120 MDa (Hetzer et al., 2005; Tran and Wentz, 2006). A nuclear pore is constructed from ~30 different proteins called nucleoporins and this number is roughly the same for yeast and higher eukaryotes (Cronshaw et al., 2002; Rout et al., 2000). All nucleoporins were previously shown to be present in 8, 16 or 32 copies per NPC consistent with the structural symmetry.

According to a structure prediction by Devos and collaborators, yeast nucleoporins contain at least one of eight different fold types and have different structural and functional roles in the nuclear pore complex (Devos et al., 2006): nucleoporins with transmembrane helices and cadherin folds localize to the exterior of the central framework and are thought to anchor the NPC to the nuclear envelope. The nucleoporins that are predicted to form the structural scaffold of the NPC are composed of  $\alpha$ -solenoid or  $\beta$ -propeller fold domains. A large group of 12 of the ~30 nucleoporins in yeast contains repeats of phenylalanine-glycine motifs (FG repeats), often in combination with coiled-coil domains that might anchor these nucleoporins to

the NPC. FG nucleoporins are suggested to line the pore channel and extend their natively unfolded FG repeat domains into the channel. These domains form a selective permeability barrier and are thought to be transiently interacting with transport factors during their passage through the pore channel (Denning et al., 2003; Frey and Görlich, 2009; Grant et al., 2003; Miao and Schulten, 2009).



**Figure 2 Distribution of nucleoporins of the nuclear pore complex**

Depicted are nucleoporins that contain phenylalanine-glycine (FG) repeats. FG repeats are thought to fill the pore channel and extend somewhat to the nucleoplasm and the cytoplasm. Note that Nup2 can asymmetrically localize to the nuclear or the cytoplasmic side (see text). Figure adapted from Stewart, 2007a.

Some of the nucleoporins containing FG repeats are distributed symmetrically on the cytoplasmic and the nuclear side of the nuclear pore complex while others reside exclusively on one side (Fig. 2; reviewed in Stewart, 2007a). FG repeats of certain nucleoporins were shown to have an important function as docking and interaction sites for various general transport factors (Isgro and Schulten, 2007).

Further functions of certain nucleoporins in nucleocytoplasmic transport have been revealed in detail (reviewed in Tran and Wente, 2006): nucleoporins positioned on the nuclear side of the NPC have functions in mRNP quality control (Nup60; Galy et al., 2004) or recruitment of the mRNA export factor Sac3 to NPCs (Nup1; Fischer et al., 2002; see below). The localization of Gle1 and Dbp5, two factors involved in mRNP remodelling (see below), at the cytoplasmic face of the NPC by interaction with Nup42 and Nup159 could assist to generate directionality on mRNP transport (reviewed in Stewart, 2007b). Another yeast nucleoporin Nup2 was found to localize to the cytoplasmic or the nuclear side of the NPC, dependent on its interaction with the GTP-bound form of the small GTPase Ran (RanGTP; see below). The dynamic localization of Nup2 (Dilworth et al., 2001) together with the involvement of this

nucleoporin in import termination (Hahn et al., 2008) could participate to ensure directionality of nuclear import through the nuclear pore channel.

## **1.2 Transport through nuclear pore complexes**

Nuclear pore complexes have to provide a high rate of macromolecular transport while maintaining high selectivity at the same time. The rate of transport through a single NPC can add up to nearly 100 MDa per second (Ribbeck and Görlich, 2001).

Various models for a conceivable mechanism of facilitated transport across the nuclear membrane are proposed and discussed extensively (reviewed in Weis, 2007). All models currently under discussion share the formation of a selective barrier by a meshwork of FG repeats that selectively restricts passage to macromolecules. The phenylalanine-glycine repeats (FG repeats) are natively unfolded structures that are present in nucleoporins that line the NPC channel and extend into its interior (Denning et al., 2003; Miao and Schulten, 2009; Tran and Wente, 2006). Passage through this meshwork of unstructured polypeptides by diffusion is limited to molecules smaller than 30-40 kDa (Ribbeck and Görlich, 2001) or ~2.5nm in radius (Mohr et al., 2009). Molecules of a greater molecular mass can only efficiently cross this barrier when bound to transport receptors that transiently interact with FG repeats. All nucleocytoplasmic transport receptors form a complex with their cargo and cross the nuclear pore channel by transient interactions with FG repeats inside the channel (Peters, 2009).

Transport receptors can generally be classified by the direction of cargo transport that they help to accomplish (nuclear import or nuclear export) and differ in the way they mediate translocation of their substrates across the nuclear membrane.

### **1.2.1 Karyopherin-dependent nucleocytoplasmic transport mechanisms**

Nuclear transport receptors that are carrying various kinds of macromolecules through NPCs are called karyopherins (meaning nuclear carrier; Radu et al., 1995) and belong to the importin  $\beta$ -like family of proteins, named after the first transport receptor that was identified to be mediating import into the nucleus (Görlich et al., 1994). The 14

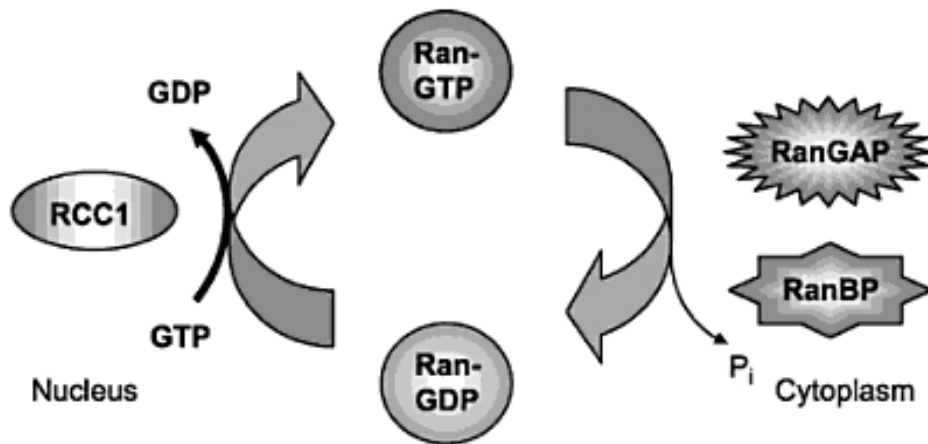
proteins from this family in yeast (>20 in mammals) are responsible for the majority of macromolecular transport across the nuclear membrane. Of the 14 identified karyopherins in yeast, nine were shown to mediate nuclear export, four work in nuclear import and one functions in both import and export (Caesar et al., 2006; Mosammaparast and Pemberton, 2004; Pemberton and Paschal, 2005). The transport factors are usually named according to the process they are involved in, exportins in export from the nucleus and importins in nuclear import.

Karyopherins exhibit structural homology of their N-terminal parts. These contribute to binding to the small GTPase Ran in complex with GTP (RanGTP; Cook et al., 2007). The C-terminal parts are responsible for selective interaction with certain targeting signals in their cargo molecules.

The processes of import into and export from the nucleus share certain common features but differ in the way that cargo is bound and released. The transport process starts when a cargo is bound by its corresponding karyopherin. The karyopherin mediates transport of its cargo across the nuclear membrane before it is finally released in the target compartment. The main difference between the properties of nuclear import and export consists of the binding to and release of the cargo from the karyopherin. These steps are regulated by and depend on interaction of the karyopherin with the small protein Ran in its GTP-bound form (RanGTP),.

#### *1.2.1.1 The Ran cycle in nucleocytoplasmic transport*

The small 25kDa GTPase Ran is essential for nucleocytoplasmic transport processes involving karyopherins. In the cell, Ran exists in two different forms, bound to the nucleotide GTP (RanGTP) or to GDP (RanGDP). Binding of RanGTP to karyopherins is needed for directional nucleocytoplasmic transport and a concentration gradient of the two forms between the nucleus and the cytoplasm is maintained by the following mechanism.



**Figure 3 The Ran cycle**

Gradients of RanGDP (higher in the cytoplasm) and RanGTP (higher in the nucleus) are provided by the depicted mechanisms and factors. RCC1 bound to chromatin in the nucleus acts as a RanGEF (guanine nucleotide exchange factor), turning RanGDP into RanGTP. RanGAP localizes to the cytoplasmic face of NPCs and with the help of Ran-binding proteins (RanBP) induces GTP hydrolysis changing RanGTP to RanGDP. Adapted from Jeon, 2005.

The two forms of Ran, RanGTP and RanGDP, can be converted into each other through the action of additional proteins in the Ran cycle (Fig. 3). RanGTP is transformed into RanGDP by the action of a RanGAP (GTPase-activating protein) and the Ran-binding protein RanBP1. RanGAP and RanBP1 are situated at the cytoplasmic side of the NPC (Mahajan et al., 1997) and can stimulate the weak GTPase activity of Ran by the factor  $10^5$  (Bischoff and Görlich, 1997; Bischoff et al., 1994; Bischoff et al., 1995). Upon entry into the nucleus, RanGDP is converted into RanGTP by the RanGEF (guanine nucleotide exchange factor) RCC1, finalizing the Ran cycle (reviewed in Pemberton and Paschal, 2005). RCC1, as well as its yeast homologue Prp20 (Kadowaki et al., 1993), associates with chromatin by interaction with histones H2A and H2B, thus securing its nuclear localization (Bischoff and Ponstingl, 1991; Nemergut et al., 2001).

To maintain the directionality and efficiency of nucleocytoplasmic transport it is crucial that the concentrations of RanGTP and RanGDP in the nucleus and the cytoplasm show a distinct gradient. This gradient is provided by the different localization of the accessory proteins RanGEF (nuclear) and RanGAP (cytoplasmic). The resulting concentration gradient of RanGTP and RanGDP serves as a cargo-binding or -release signal for karyopherins (see above) and ensures the directionality of nucleocytoplasmic transport. Additionally, the concentration of Ran itself is higher



in the nucleus than in the cytoplasm because RanGDP is transported from the cytoplasm into the nucleus by the small protein NTF2 (nuclear transport factor; Ribbeck et al., 1998).

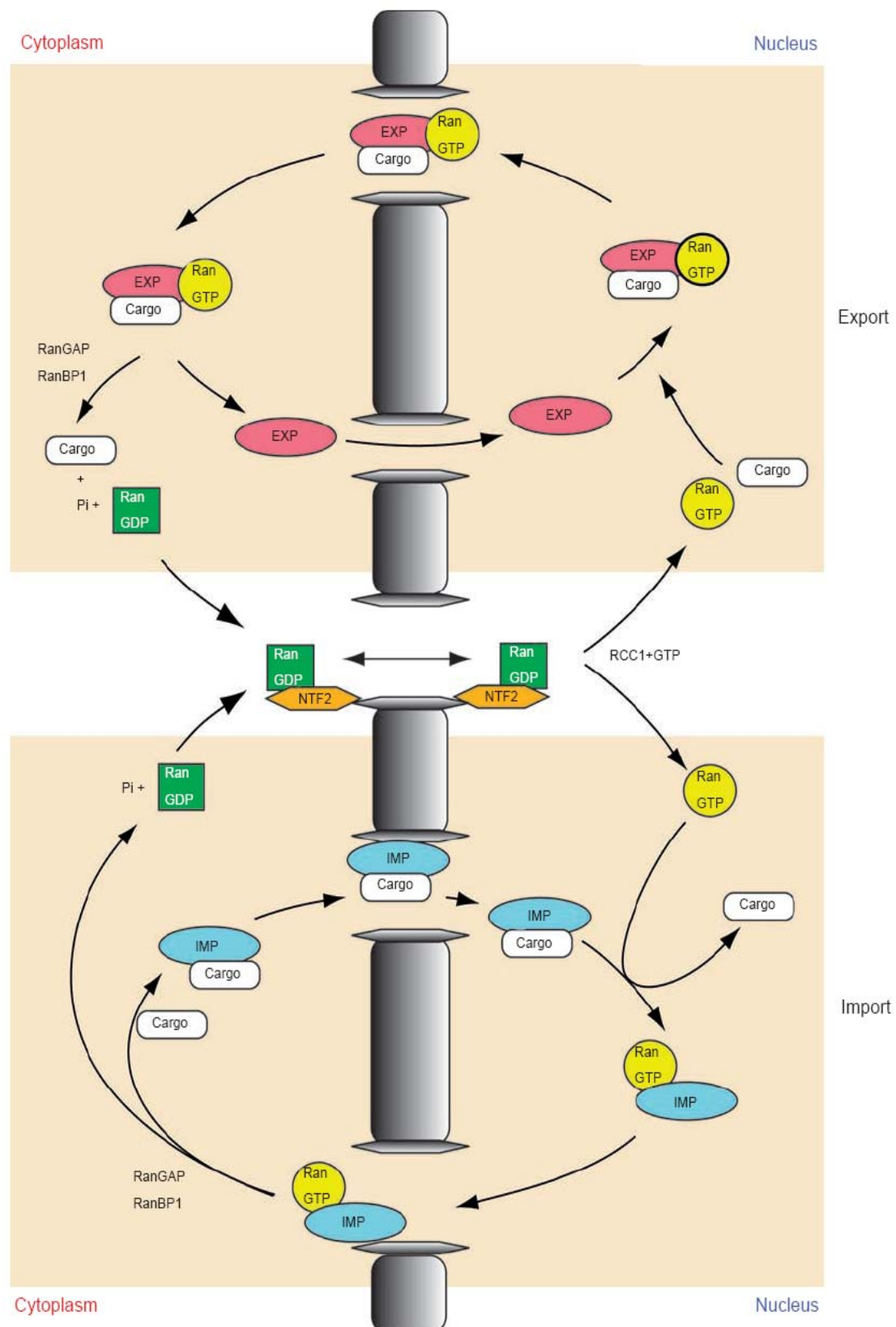
#### *1.2.1.2 Karyopherin-dependent import into the nucleus*

The majority of cargos for nucleocytoplasmic transport are mRNAs (exported to the cytoplasm) and nuclear proteins (imported into the nucleus). While the nuclear export of mRNA constitutes a different mechanism without involving karyopherins (see below), the import of soluble proteins into the nucleus follows the classical import route. Initially, the nuclear localization signal (NLS) of a cargo protein is recognized either directly by an importin  $\beta$  protein or through the adaptor protein importin  $\alpha$ . In the classical import pathway, importin  $\alpha$  binds to the NLS cargo and forms a transport-competent heterodimer with importin  $\beta$  (Goldfarb et al., 2004). The classical NLS for import of proteins into the nucleus is usually a short stretch that contains either one cluster of basic amino acids or a bipartite motif of two basic clusters separated by a linker of 10-12 residues (reviewed in Hahn et al., 2008). Next, the ternary NLS cargo:importin  $\alpha$ :importin  $\beta$  complex moves through the pore by transient interactions with the FG repeats inside the pore channel. The exact process of translocation through the NPC is still poorly understood (see above and in Fried and Kutay, 2003; Weis, 2007) but it is clear that protein cargo generally traverses the nuclear membrane in a folded conformation, contrary to mitochondrial import (Görlich and Kutay, 1999). Binding of RanGTP in the nucleus subsequently induces a structural change in importin  $\beta$  that leads to dissociation of the NLS cargo (Cook et al., 2007), thus finalizing the import process.

#### *1.2.1.3 Karyopherin-dependent export from the nucleus*

The range of cargos for export from the nucleus into the cytoplasm includes proteins shuttling between the nucleus and the cytoplasm and different types of RNA such as tRNA, rRNA assembled in ribosomal subunits and mRNA. The exportin Cse1 (or CAS in humans) is responsible for the export of only one cargo, importin  $\alpha$  (Kutay et al., 1997; Solsbacher et al., 1998; reviewed in Cook et al., 2007). Due to the significance of importin  $\alpha$  for nuclear import, blocking its export by deleting Cse1

cannot be tolerated by the cell and leads to cell death. A second exportin with a whole plethora of export substrates is Crm1 (or exportin 1). More than 75 potential export cargos for Crm1 carrying the leucine-rich recognition motif called nuclear export signal (NES; Fornerod et al., 1997; Kosugi et al., 2008; Wen et al., 1995) could be identified (la Cour et al., 2003). Recently, the structure of Crm1 in complex with RanGTP and its cargo snurportin (an import adapter for small nuclear ribonucleoproteins, snRNPs) was solved (Dong et al., 2009; Monecke et al., 2009). This structure indicates that Crm1 recognizes and binds its cargo through a hydrophobic cleft accommodating an N-terminal, hydrophobic and NES-like stretch of the cargo protein. Crm1 recognizes its NES-cargo in the presence of RanGTP, thus initiating the export process. After the formation of the Crm1: RanGTP: cargo export complex, it is then translocated to the cytoplasm through the nuclear pore channel where GTP is hydrolyzed and Crm1, RanGDP and the cargo dissociate from each other (Matsuura and Stewart, 2004; Nigg, 1997; Ohno et al., 1998). The differential role of RanGTP or RanGDP in the processes of nuclear import and export likely contributes to the directionality of nucleocytoplasmic transport. An overview of the processes of karyopherin-dependent nucleocytoplasmic transport is shown in Fig. 4.



**Figure 4 The Importin-Exportin cycle**

The export of cargo from the nucleus and the return of its exportin to the cytoplasm is depicted in the upper part, import of cargo from the cytoplasm into the nucleus and recycling of the respective importin in the lower part. For a more detailed account of the Ran cycle, see Fig. 3. IMP – Importin; EXP – Exportin; from Hill, 2009.

Similar to the export of protein cargo, tRNA transcripts form a ternary complex with RanGTP and the specific tRNA exporter exportin-t in the nucleus which dissociates upon GTP hydrolysis in the cytoplasm (Kutay et al., 1998). The structure of exportin-t from *Schizosaccharomyces pombe* (Xpot) alone and in complex with RanGTP and a tRNA could recently be solved, revealing new mechanistic details of this complex formation (Cook et al., 2009). Structural and biochemical data from this study indicate that Xpot associates with RanGTP before tRNA can be bound by the Xpot-RanGTP heterodimer, indicating a defined order of complex formation. In the ternary complex, Xpot contacts the 5' and 3' ends of a mature tRNA, ensuring the correct processing of a tRNA before export can occur and thus connecting tRNA export to quality control.

Interestingly, the *Saccharomyces cerevisiae* exportin-t homologue Los1 is not essential for the cell (Hurt et al., 1987). This indicates the existence of an alternative route for tRNA export. A linkage of tRNA export to tRNA aminoacylation was suggested as Los1 was shown to become essential when the function of a member of the aminoacylation machinery (Arc1) is perturbed (Simos et al., 1998). Other studies also connect Los1-independent export of a subset of tRNAs to aminoacylation although the export receptor for this pathway remains to be determined (Feng and Hopper, 2002; Grosshans et al., 2000). An additional factor involved in tRNA export, Cex1, was recently shown to channel aminoacylated tRNAs from the NPC to the ribosomal elongation factor eEF1 $\alpha$  (McGuire and Mangroo, 2007) and thus to make exported tRNAs available for translation.

Ribosome biogenesis and the export of pre-ribosomal subunits are complex and highly regulated processes. At this point, not much is known about the export mechanism of the 40S subunit but it seems clear that it involves the exportin Crm1 and depends on a functional Ran cycle (Moy and Silver, 1999). Export of the large 60S ribosomal subunit is better understood: the classical exportin Crm1 can bind to the pre-ribosomal subunit through the NES-containing adaptor Nmd3 and assists export in a RanGTP-dependent manner (Gadal et al., 2001). Recent findings from our laboratory could show that two other nuclear export receptors are involved in the export of the large pre-ribosomal particle. One of them, Arx1, is a rather unique export receptor as it does not show structural homology to any of the known transport receptors but to methionine aminopeptidases. In contrast to these, Arx1 does not have enzymatic activity and its central cavity does not interact with methionine. Instead,

the central cavity of Arx1 is predicted to be involved in binding to the late 60S particle and FG repeats, possibly facilitating the passage of the 60S particle through the nuclear pore (Bradatsch et al., 2007). A third export receptor for the 60S pre-ribosomal particle was found to be the heterodimer Mex67-Mtr2, the general export receptor for mRNPs (see below). Mex67-Mtr2 contains a previously uncharacterized interaction surface with the pre-60S particle in the middle, NTF2-like domain (Yao et al., 2007). This surface is characterized by loop insertions which are important for pre-60S export and absent from the human homologue of Mex67-Mtr2, TAP-p15. Positively charged amino acids inside the loop insertions were shown to mediate the association of Mex67-Mtr2 with the pre-60S particle, presumably through direct contacts with the 5S rRNA. Additionally, the loop insertions of Mex67-Mtr2 could be shown to harbour a binding site for a structural nucleoporin that does not contain FG repeats (Yao et al., 2008). This interaction was shown to be required for efficient mRNA export and demonstrates a dual role for this surface of Mex67-Mtr2 in pre-60S and mRNA export from the nucleus.

While the majority of nucleocytoplasmic transport of macromolecules involves karyopherins and a RanGTP gradient, there are examples for transport independent of the mechanisms described above. An important and well-studied example for a karyopherin-independent mechanism is the export of mRNAs assembled into mRNPs (messenger ribonucleoprotein particles) from the nucleus using a general mRNA export receptor that is structurally different from karyopherins (Herold et al., 2000).

### **1.2.2 Karyopherin-independent export of mRNA from the nucleus**

The majority of mRNAs is exported from the nucleus independent of karyopherin  $\beta$  proteins or Ran. mRNA export is a complex process involving many RNA-binding and modifying proteins necessary to form export-competent mRNPs that can be translocated to the cytoplasm.

#### *1.2.2.1 mRNP composition and assembly*

The formation of an mRNP that is eventually exported from the nucleus is closely linked to the transcription of a nascent mRNA transcript. mRNAs are transcribed by

the RNA polymerase II as pre-mRNAs that become co- and post-transcriptionally modified. Modifications of the mRNA include the addition of 7-methylguanosine ( $m^7G$ ) to the 5' end (5' capping), cleavage of the 3' end and subsequent addition of ~200 adenine nucleotides in yeast (3' polyadenylation) and removal of non-coding introns by splicing (Cheng et al., 2006; Cole and Scarcelli, 2006; Luna et al., 2008; Saguez et al., 2005). During the processing steps and packaging of the transcript into an mRNP, proteins involved in the mRNP export pathway are recruited to the particle. Among these are certain factors of the exon-junction complex (EJC) and SR proteins (for serine-arginine rich proteins) that assemble with the mRNA during splicing (Huang et al., 2003; Tange et al., 2004) and accompany the mRNP to the cytoplasm (Caceres et al., 1998; Dreyfuss et al., 2002; Windgassen et al., 2004). One of these factors in yeast, the protein Npl3, associates with the mRNA early in the transcription process (Lei et al., 2001) and was suggested to recruit the export receptor Mex67 to the nascent transcript. Upon exit from the nucleus, Npl3 is phosphorylated by the cytoplasmic kinase Sky1 and causes disassembly of the mRNP (Gilbert et al., 2001; Siebel et al., 1999; Yun and Fu, 2000). Phosphorylated Npl3 is subsequently reimported into the nucleus and recycled by dephosphorylation by Glc7 in the nucleus (Gilbert and Guthrie, 2004). An additional factor escorting the mRNP through the NPC is the DEAD-box helicase Dbp5, thought to dismantle the mRNP and release it from the cytoplasmic side of the pore, thus conferring directionality to mRNP export (Tran et al., 2007; reviewed in Stewart, 2007b). Dbp5 is recruited to the mRNP in the nucleus (Cole and Scarcelli, 2006) and exhibits RNA helicase activity that is activated by Gle1, a factor located on the cytoplasmic side of the NPC, and its cofactor IP<sub>6</sub> (inositol hexakisphosphate; Alcazar-Roman et al., 2006; Weirich et al., 2006). The mRNP remodelling activity of Dbp5 and active displacement of the poly (A)-binding protein Nab2 from the mRNP at the cytoplasmic side of the NPC (Tran et al., 2007) could be important for unidirectional transport and release of the mRNP from the NPC.

An important process which is closely connected with and necessary for efficient mRNP formation and export, is the quality control of mRNPs. The quality control machinery monitors the correct assembly of mRNPs and makes sure that improperly processed or assembled mRNPs are retained in the nucleus and targeted for degradation (Schmid and Jensen, 2008). Surveillance steps take place throughout the generation and assembly of an mRNP, very early by exosome recruitment to nascent

pre-mRNPs (Andrulis et al., 2002; Hessle et al., 2009) as well as just before exit of the mRNP by the NPC-anchored Mlp1 protein which was shown to be important for the retention of unspliced mRNAs in the nucleus (Galy et al., 2004). An additional involvement of Mlp1 in the mRNA export process by interaction with the mRNA binding protein Nab2 was recently identified and found to be essential for mRNA export (Fasken et al., 2008). The dual role of Mlp1 is a good example of the interplay between different steps and processes involved in mRNA export mediated by the general export receptor Mex67-Mtr2 (reviewed in Luna et al., 2008).

#### *1.2.2.2 The general mRNA export receptor Mex67-Mtr2*

The heterodimer Mex67-Mtr2 (functionally homologous to metazoan TAP-p15) was first identified in yeast as the general mRNA export receptor to be necessary for export of nuclear mRNA through nuclear pore complexes (Grüter et al., 1998; Santos-Rosa et al., 1998; Segref et al., 1997). The general mRNA export receptors, in yeast as well as in metazoans, consist of an amino-terminal (N) domain, a leucine-rich repeat (LRR) domain, an NTF2-like middle (M) domain and a UBA-like carboxy-terminal (C) domain (Katahira et al., 1999; Santos-Rosa et al., 1998; Segref et al., 1997).

The N-terminal and LRR domains are important for the interaction with mRNA cargoes mediated by the RNA-binding protein Yra1 (see below and Strässer and Hurt, 2000). The middle domain of Mex67 and its binding partner Mtr2 are both structurally related to the fold of NTF2 (Fribourg and Conti, 2003; Senay et al., 2003), the import factor for RanGDP (see above). Mex67 and Mtr2 interact with each other through their NTF2-like domains and both NTF2-like domains were shown to interact with FG repeat nucleoporins (Fribourg et al., 2001; Santos-Rosa et al., 1998; Strässer et al., 2000). Additional functions of yeast-specific loop insertions in the NTF2-like domains of Mex67 and Mtr2 in the export of the 60S preribosomal subunit have recently been demonstrated (see above and Yao et al., 2008; Yao et al., 2007).

The C-terminal part of Mex67 has a UBA-like (ubiquitin-associated) fold that was shown to interact with nucleoporin FG repeats (Grant et al., 2003) as well as with Hpr1, a member of the TREX/THO complex involved in coupling of transcription and mRNA export (Hobeika et al., 2009). The FG repeat-binding domains situated in Mtr2 and the NTF2-like middle domain as well as in the C-terminal UBA domain of

Mex67 are necessary for transport of cargo through the nuclear pore and shuttling of the mRNA export receptor between the nucleus and the cytoplasm (Hobeika et al., 2009; Terry and Wente, 2007).

Mex67-Mtr2 accomplishes its classical role as the main export receptor for mRNPs with the significant contribution of the TREX complexes (TREX and TREX-2), connecting transcription and mRNA export.

#### *1.2.2.3 The role of the TREX complex in mRNA export*

The export receptor Mex67-Mtr2 can only fulfil its role in mRNA export efficiently when it is targeted to nascent transcripts by adaptor proteins.

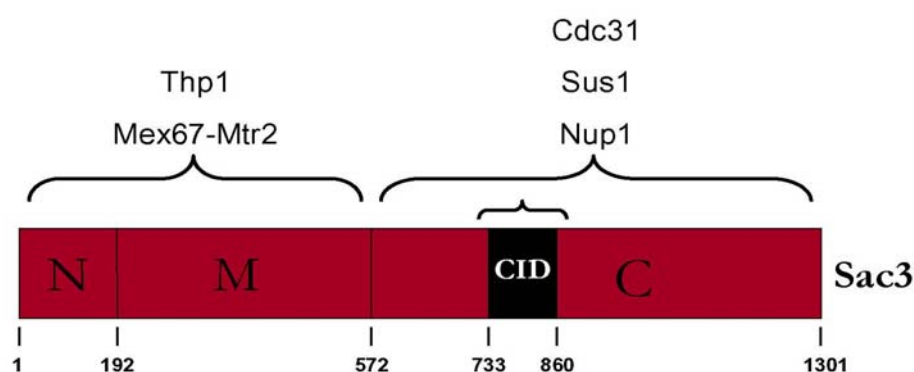
One of these adaptors is Yra1, an RNA-binding protein shown to be recruited to actively transcribed genes (Abruzzi et al., 2004; Lei et al., 2001; Zenklusen et al., 2002) by interaction with an RNA polymerase II-associating factor, Pcf11 (Johnson et al., 2009) and the DEAD box RNA helicase Sub2 (Strässer and Hurt, 2001). Yra1 and Sub2 interact with the THO complex to be targeted to transcribing genes and nascent mRNAs. This complex consists of the four proteins Tho2, Hpr1, Mft1, and Thp2, and was reported to be involved in transcription elongation, mRNP biogenesis and mRNA export (Chavez et al., 2000; Hobeika et al., 2009; Jimeno et al., 2002; Rondon et al., 2003; Rougemaille et al., 2008; Strässer et al., 2002). The export factors Yra1 and Sub2 associate with the THO complex to form the larger TREX complex (together with Tex1; Strässer et al., 2002) and are subsequently loaded onto the early mRNP. After Yra1 and Sub2 bind to the mRNP, the export receptor Mex67-Mtr2 is recruited to the particle through binding to Yra1 (Strässer and Hurt, 2000) and most likely displaces Sub2 from the mRNA as both Sub2 and Mex67-Mtr2 were shown to interact with the same domains of Yra1 (Strässer and Hurt, 2001). Mex67-Mtr2, bound to the mRNP, subsequently assists in exporting the mRNA through the nuclear pore channel. Yra1 (Aly or REF1 in metazoans) as well as Sub2 (UAP56) are conserved in higher eukaryotes and their homologues were found to link splicing to mRNA export rather than transcription as in yeast (Luo et al., 2001; Masuda et al., 2005; Zhou et al., 2000). This might be due to a higher percentage of intron-containing genes dependent on the splicing machinery in higher eukaryotes than in yeast.



An additional role of the THO/TREX complex for genomic stability was analyzed in mutants of subunits of the complex. Disrupting the function of the THO/TREX complex by mutation of single components was shown to lead to DNA-RNA hybrid formation (R loops; Huertas and Aguilera, 2003) involving nascent mRNAs. Disturbed release of the mRNP from the chromatin template and incorrect assembly of the nascent mRNAs into mRNPs could make them available for DNA-RNA hybrid formation, leading to reduced transcription efficiency and genome instability (Jimeno et al., 2002; Libri et al., 2002; Tous and Aguilera, 2007; Wellinger et al., 2006). Thus, a functional interplay of transcription and export of correctly assembled mRNPs is necessary for genome stability and efficient delivery of translation-competent mRNPs to the cytoplasm.

#### 1.2.2.4 The role of the TREX-2 complex in mRNA export

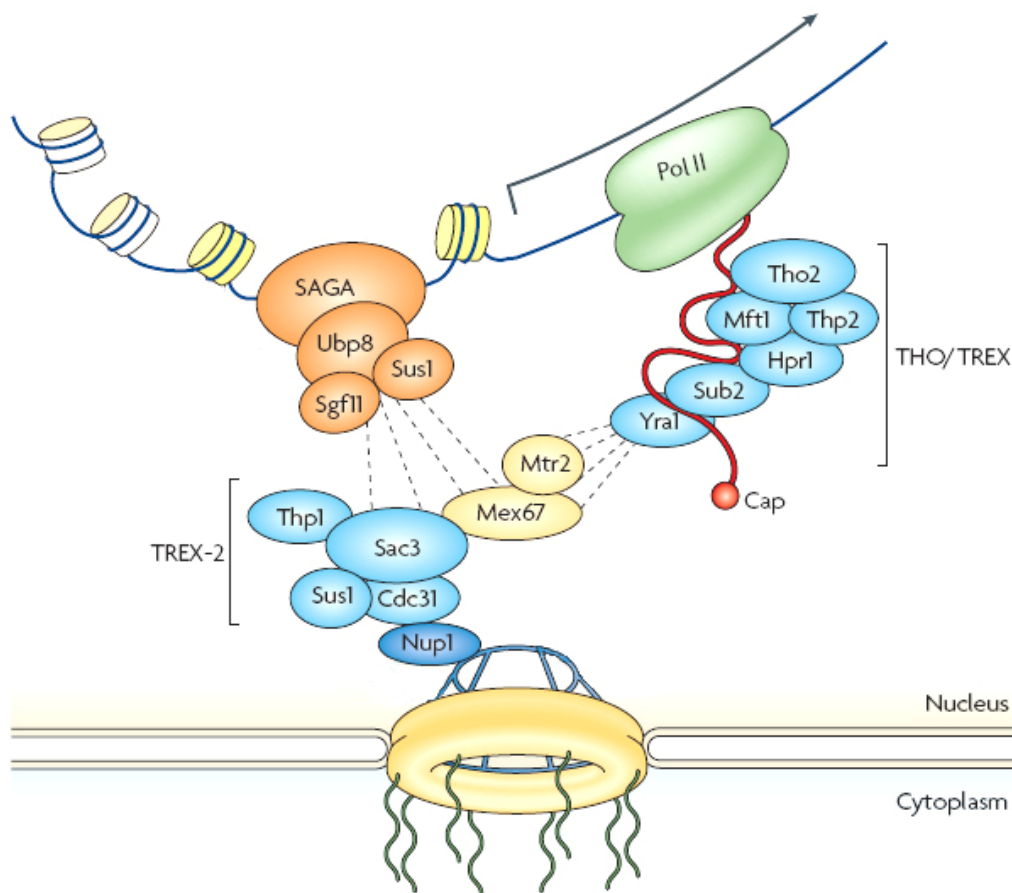
A second complex important for efficient mRNA export is represented by the TREX-2 complex, alternatively termed Sac3-Thp1-Cdc31-Sus1 complex (Fischer et al., 2002; Köhler and Hurt, 2007). The complex was found to assemble around the protein Sac3 which was originally identified as a suppressor of an actin mutation (Novick et al., 1989) and reported to be associated with leucine transport (Stella et al., 1999). Sac3 is located at the nuclear side of the NPC by interaction with the nuclear basket protein Nup1 and directs mRNPs for export in cooperation with Mex67-Mtr2 (Fischer et al., 2004; Fischer et al., 2002; Gallardo et al., 2003).



**Figure 5 Overview of the domains of Sac3 and their binding partners**

The protein Sac3 can be separated in a conserved middle domain and flanking N-terminal and C-terminal regions. Proteins depicted to be interacting with the C-terminal region were shown to bind to the CID (Cdc31-interacting domain) motif (see text). The boundaries are annotated below the sequence, numbers for the CID motif from Fischer et al., 2002.

The interaction with the general export receptor Mex67 and Thp1 is mediated by N-terminal and middle regions (N+M domain) of Sac3, while the C-terminus was shown to be necessary for localization to the nuclear pore complex (Fischer et al., 2002; Fig. 5). An additional putative member of the TREX-2 complex, Sem1, was recently shown to associate with Sac3 and Thp1 and might facilitate interaction of Sac3 and Thp1 with each other (Faza et al., 2009). An overview of the two complexes coupling transcription and mRNA export, TREX and TREX-2, is shown in Figure 6.



**Figure 6 The TREX complex and the TREX-2 complex and their functional environment**

The individual components of the THO/TREX and the TREX-2 complexes are depicted with their interactions towards chromatin-interacting complexes, the general mRNA exporter Mex67-Mtr2 and the nuclear pore complex (for TREX-2). Dotted lines represent predicted interactions between complexes. Adapted from Köhler and Hurt, 2007.

A distinct C-terminal region of Sac3 termed the CID motif (for Cdc31-interacting domain) was identified as the platform for nuclear pore localization of the whole complex as well as for the interaction with the remaining TREX-2 members Cdc31 and Sus1 (Fischer et al., 2004; Fig. 6). Cdc31 as well as Sus1 fulfil additional roles to

their TREX-2 functions in the cellular context. While Cdc31 is involved in the duplication of microtubule-organizing centers (Spang et al., 1993; reviewed in Helfant, 2002), Sus1 was originally found to be a functional member of TREX-2 as well as the transcriptional coactivator SAGA (Spt-Ada-Gcn5 acetyltransferase) which mediates histone acetylation and deubiquitination (Rodriguez-Navarro et al., 2004). Inside SAGA, Sus1 forms a deubiquitinating module (DUB module) with Ubp8 and Sgf11 which is anchored to SAGA by Sgf73 and mediates deubiquitination of histone H2B (Köhler et al., 2008; Zhao et al., 2008). Correct formation of the DUB module is essential for deubiquitination of H2B by the ubiquitin-specific protease Ubp8 which is in turn required for optimal transcriptional activation and regulation (see below and Daniel et al., 2004; Henry et al., 2003; Köhler et al., 2006; Köhler et al., 2008; Wyce et al., 2007). Performing these two functions, Sus1 was suggested to form a bridge between SAGA-dependent transcription initiation and the mRNA export machinery at the nuclear side of NPCs (Rodriguez-Navarro et al., 2004).

A recent study from Jani and collaborators (Jani et al., 2009) revealed the atomic structure of the central scaffold Sac3<sup>CID</sup> bound to Cdc31 and Sus1 (see below). This study narrowed the boundaries of the interaction platform CID further and showed that it forms an extended  $\alpha$ -helix with one copy of Cdc31 and two copies of Sus1 sequentially wrapped around the helical rod formed by Sac3<sup>CID</sup> in the complex. Deletions of distinct binding surfaces of the CID motif for its partner proteins show that disruption of Sus1 or Cdc31 binding to Sac3<sup>CID</sup> can abolish NPC positioning and inhibit mRNA export, albeit to different extents (see Discussion and Jani et al., 2009). Notably, the presence of orthologues of Sac3, Cdc31 and Sus1 in higher eukaryotes suggests that this interaction platform might be conserved. In fact, a putative CID motif could be identified in the human Sac3 orthologue GANP and was able to bind to ENY2, the human Sus1 orthologue, *in vitro* (Jani et al., 2009). Similar results were previously obtained for the orthologues of Sac3 (Xmas-2) and Sus1 (E(y)2) in *Drosophila* and, in analogy to their counterparts in yeast, both proteins localize to the NPC and function in mRNA export (Kurshakova et al., 2007).

On all accounts, the Sac3<sup>CID</sup> motif is a central platform inside the yeast TREX-2 complex which has an important role in the integration of SAGA-dependent transcription and Mex67-dependent mRNA export.

### 1.3 Coupling of TREX-2 to the transcriptional coactivator SAGA

The various processes taking place from the transcription initiation of a given gene to the passage of its mRNA transcript through the nuclear pore channel were shown to be functionally and physically coupled. There is a complex network of regulation and crosstalk between these processes which is necessary for efficient mRNP formation and export (reviewed in Luna et al., 2008; Maniatis and Reed, 2002).

Lately, repositioning of genes to the nuclear periphery was shown to have an important role in the regulation and integration of transcription and mRNA export. The transcriptional coactivator complex SAGA and the mRNA export complex TREX-2 were concomitantly shown to be involved in this process and necessary for efficient transcription-coupled mRNA export from the nucleus.

#### 1.3.1 The concept of gene gating

The distribution of chromosomes in the nucleus is known to be non-random and they are organized in so-called “chromosomal territories” in higher eukaryotes (Lamond and Earnshaw, 1998). These chromosomal territories seem to be absent in *S. cerevisiae*, however genomic loci could be shown to localize to so-called “gene territories” in yeast that become remodelled upon transcriptional activation (Berger et al., 2008). In the context of territorial localization of active and inactive genes, the nuclear periphery was thought to be connected with transcriptional silencing (Andrulis et al., 1998; Cockell and Gasser, 1999). This might be true for regions of constitutive heterochromatin like telomeres or other silenced loci that can become statically tethered to the nuclear periphery mediated by the heterochromatin-binding SIR complex in yeast (Ahmed and Brickner, 2007; Taddei et al., 2009; Towbin et al., 2009). However, recent developments seem to nurture an earlier hypothesis of so-called “gene gating” (Blobel, 1985). This hypothesis stated that the non-random distribution of chromatin in the nucleus results from the specific interaction of active chromatin regions with a nuclear pore complex which would gate a given gene and its transcripts to a single NPC in the nuclear membrane. This model might not apply to all genes but recent findings largely comply with the gene gating postulate: Various studies demonstrated that a whole set of genes was shown to rapidly reposition

towards the nuclear periphery upon transcriptional activation (Brickner and Walter, 2004; Cabal et al., 2006; Casolari et al., 2005; Dieppois et al., 2006; Drubin et al., 2006; Taddei et al., 2006). Repositioning to NPCs seems to be functionally coupled with the mRNA export machinery as it was shown to require the mRNA export receptor Mex67 (Dieppois et al., 2006) as well as the TREX-2 subunits Sac3 and Sus1 (Cabal et al., 2006). The recruitment of genes to the nuclear periphery and their tether to NPCs was reported to depend on the promoter (Dieppois et al., 2006) or 3'UTR sequences (Abruzzi et al., 2006; Taddei et al., 2006).

Dynamic repositioning to the vicinity of the NPC seems to be connected to transcriptional activation (see above) while the regions that are more statically bound to regions in between nuclear pore complexes tend to be silenced (reviewed in Towbin et al., 2009). These observations indicate that the connection between NPC positioning and gene activation, as suggested by the gene gating hypothesis, might serve as a general mechanism in eukaryotic cells.

### **1.3.2 SAGA-dependent transcriptional activation**

The SAGA complex (Spt-Ada-Gcn5 acetyltransferase) is a modular ~1.8MDa multiprotein complex with diverse roles in transcriptional activation like recruitment of the transcription machinery to activated genes and chromatin remodelling (reviewed in Daniel and Grant, 2007). Remodelling of chromatin in the course of transcriptional activation mediated by SAGA includes histone acetylation and deubiquitination in promoter and coding regions of target genes (Govind et al., 2007; Grant et al., 1997; Huisinga and Pugh, 2004; Wyce et al., 2007). In yeast, SAGA is one of four complexes containing the histone acetyltransferase Gcn5 as a catalytic subunit (Lee and Workman, 2007). Acetylation of lysine residues of histone H3 by Gcn5 in promoter and coding regions of genes induces nucleosome eviction and transcriptional activation of the respective gene (Govind et al., 2007). These acetyl-lysines can subsequently be recognized by a so-called bromodomain, a region present in Gcn5 and other factors that mediate chromatin remodelling and transcriptional activation (Mujtaba et al., 2007). In the case of SAGA, the bromodomain of Gcn5 serves to retain the SAGA complex at acetylated histones H3 to cooperatively acetylate nucleosomes to fully activate affected genes (Li and Shogren-Knaak, 2009)

An additional function of SAGA lies in the deubiquitination of ubiquitinated histone H2B by the ubiquitin protease Ubp8 as part of the deubiquitinating (DUB) subcomplex of SAGA (Ingvarsdottir et al., 2005; Köhler et al., 2006; Lee et al., 2005; Shukla et al., 2006). The integrity of the DUB module containing Sus1, Sgf11, Sgf73 and Ubp8 was shown to be important for correct deubiquitination by Ubp8 *in vivo* and *in vitro* as Ubp8 is enzymatically inactive when the module is not assembled (Köhler et al., 2008). Recent studies were able to show that the DUB module components have human orthologues which include ATXN7 (Sgf73), ATXN7L3 (Sgf11), USP22 (Ubp8) and ENY2 (Sus1; Zhao et al., 2008). Similar to the role of Ubp8 in yeast, its human homologue USP22 is responsible for histone H2B deubiquitylation. Notably, the function of USP22 was shown to be required for progression through the cell cycle and found to be linked to diverse types of cancer which could be due to the role of USP22 in transcription regulation (Zhang et al., 2008).

In yeast, efficient transcriptional activation and elongation seems to require a regulated sequence of ubiquitination of lysine 123 in histone H2B by Rad6/Bre1 (Robzyk et al., 2000; Wood et al., 2003) and removal of ubiquitin by Ubp8 (Daniel et al., 2004; Henry et al., 2003; Weake and Workman, 2008). Furthermore, the ubiquitination of H2B seems to be linked to methylation of lysine 4 of histone H3 (H3K4me), another histone modification associated with transcriptional activation (reviewed in Shilatifard, 2008). Thus, a functional DUB module is required for coordinated histone deubiquitination and subsequent steps necessary for the efficient activation of transcription.

Transcriptional coactivation involving SAGA functions was shown to be needed for full expression of heat-shock genes (Zanton and Pugh, 2004), the *PHO* genes (Barbaric et al., 2003) and other genes important for a rapid response of the cell to altered environments (Lo et al., 2001; Qiu et al., 2004; Taddei et al., 2006). The best studied among the SAGA-dependent genes are the *GAL* genes as they are easily inducible by galactose and dependent on activation by SAGA (Bhaumik and Green, 2001; Larschan and Winston, 2001). A physical link of SAGA components to NPC-bound Mlp1 was recently shown to be involved in the repositioning of *GALI* to the nuclear pore (Luthra et al., 2007).

Another link between SAGA and NPCs appears to involve the mRNA export machinery. Sus1 was reported to be a functional member of the mRNA export

complex TREX-2 as well as of SAGA (Rodriguez-Navarro et al., 2004). These findings moved the transcriptional coactivator into the center of our interest.

Unexpected new aspects connecting SAGA with the mRNA export complex TREX-2 came from a study performed in our laboratory which indicates that a region in the deubiquitinating (DUB) module component Sgf73 has a role in the association of the TREX-2 proteins Sac3 and Thp1 with SAGA (Köhler et al., 2008). Surprisingly, the dissociation of Sus1 from SAGA in a *ubp8Δ* background leaves this interaction intact, indicating that there is a TREX-2-SAGA connection that is not primarily mediated by Sus1. Still, a different study could indeed demonstrate that the TREX-2 components Sac3 and Sus1 are needed for gene repositioning and tethering of the *GALI* locus to the nuclear pore (Cabal et al., 2006).

The connection of SAGA with the mRNA export machinery involving the TREX-2 components Sac3 and Sus1 represents the coupling of two processes to recruit transcribing genes to the nuclear periphery, thus integrating transcription and mRNA export. This coupling could enhance efficient gene expression and subsequent mRNA export.

### **1.3.3 TREX-2 subunits are involved in transcription-coupled mRNA export**

The identification of Sus1 as a member of the SAGA and TREX-2 complexes (Rodriguez-Navarro et al., 2004) argued for a role of Sus1 and its binding partner in TREX-2, Sac3 (Fischer et al., 2004), in the bridging of transcription and mRNA export. In fact, both proteins were shown to be important for repositioning of the *GALI* locus to the NPC upon transcriptional activation (Cabal et al., 2006; Drubin et al., 2006). Sac3 brings different protein machineries together and contains in its CID motif a connection to NPC positioning and the interaction surface with Sus1 (see above). Yet, it remains to be shown if the function of the CID is confined to being the interaction scaffold for Cdc31 and Sus1 or if additional processes are associated with this region of Sac3.

Sus1, being a functional member of SAGA and TREX-2, represents an intriguing example of a protein with a dual function. Most interestingly, these two functions seem to be closely connected during gene activation. Sus1 was shown to be present at

the *GALI* promoter and reported to be required for transcription activation and full expression of *GALI* which is probably attributable to its role in histone deubiquitylation by the SAGA complex (Köhler et al., 2006; Rodriguez-Navarro et al., 2004; reviewed in Rodriguez-Navarro, 2009).

However, the dual function of Sus1 in SAGA and TREX-2 poses a problem for a detailed analysis of its separate roles. A complete deletion of Sus1 affects both complexes at the same time, complicating the examination of the roles of Sus1 in the separate complexes. In recent studies on functions of Sus1 in transcription elongation (Pascual-Garcia et al., 2008), genome integrity (Gonzalez-Aguilera et al., 2008) and association of active genes with the nuclear rim (Chekanova et al., 2008) it was difficult to analyze the specific role of Sus1 in TREX-2 independent of its SAGA function. Therefore, a way to study the function of Sus1 in one of its complexes (SAGA or TREX-2) without affecting the other is a prerequisite to understand the interesting and two-sided function of the small protein connecting transcription and mRNA export.



## 1.4. Aim of this work

The TREX-2 (Sac3-Thp1-Cdc31-Sus1) complex plays an important role in the export of mRNA (Fischer et al., 2002) and could couple transcription with mRNA export (Rodriguez-Navarro et al., 2004). The C-terminal CID (Cdc31-interacting domain) motif of the TREX-2 protein Sac3 acts as an interacting surface for Sus1 and Cdc31 (Fischer et al., 2004) and could integrate transcription with mRNA export.

At the beginning of my PhD thesis, I sought to analyze how TREX-2 and SAGA could functionally interact with each other by exploiting the *SAC3<sup>CID</sup>* motif to identify additional factors involved in transcription-coupled mRNA export. In a second approach that was based on a comprehensive mutational analysis of Sus1 I aimed to find out how Sus1 interacts with either TREX-2 or SAGA. Overall, these studies should further elucidate the mechanisms by which transcription and mRNA export are coupled.

## 2. RESULTS

### 2.1 Novel genetic interactions between *SAC3<sup>CID</sup>* and factors involved in transcriptional regulation

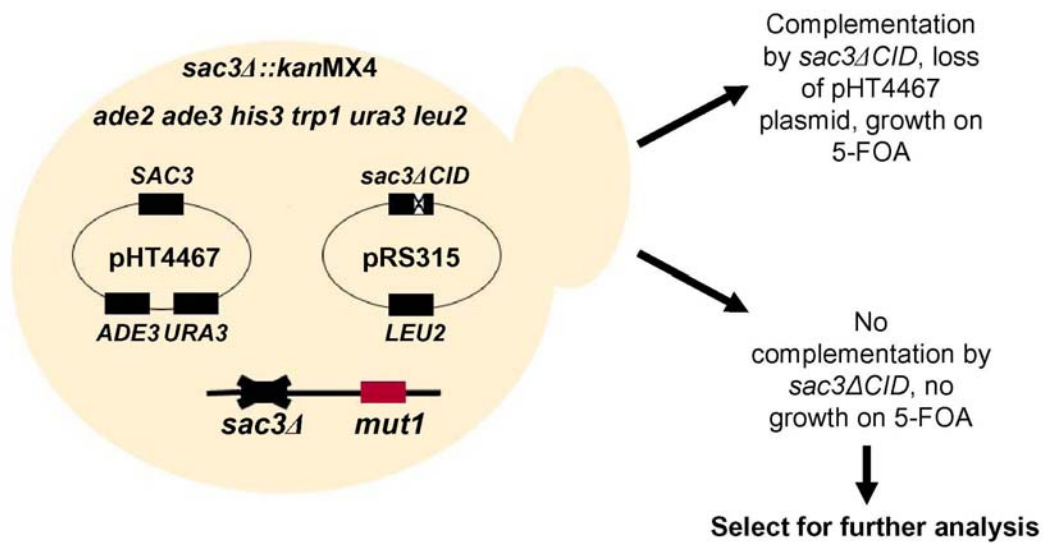
Sac3 is a central component of the mRNA export complex TREX-2 and its C-terminal CID motif was identified as the binding site for Sus1, which is also a subunit of the transcriptional co-activator SAGA and suggested to physically connect SAGA and TREX-2 (Fischer et al., 2004; Fischer et al., 2002; Rodriguez-Navarro et al., 2004). Because of the direct interaction of the CID motif with Sus1, the Sac3<sup>CID</sup> was thought to be involved in the physical coupling of TREX-2 and SAGA. Therefore, I sought to identify and analyze factors that are genetically linked to this domain and thus to transcription-coupled mRNA export.

Before I started my PhD work, a synthetic lethal (SL) screen with the *sac3* null allele (*sac3Δ*) has been performed in our laboratory by Tamas Fischer and Attila RÁCZ as previously described (Doye and Hurt, 1995; Wimmer et al., 1992). This genetic screen is based on the phenomenon of synthetic lethality which describes the genetic interaction of two nonlethal mutations that lead to cell death when combined in the same cell. In the past, synthetic lethal screen have been successfully used to identify additional factors in the genetic network of a gene of interest.

Notably, the *sac3Δ* SL screen previously led to the identification of novel genetic interactions, *e.g.* between *SAC3* and a recently identified RNA endoribonuclease involved in mRNP quality control (*SWT1*; Skruzny et al., 2009).

After the *SAC3<sup>CID</sup>* was identified as the binding site for Sus1 and Cdc31 and found to be important for nuclear pore targeting of TREX-2 (Fischer et al., 2004), I sought to rescreen the collection of *sac3Δ* SL mutants for specific genetic interactions with this motif.

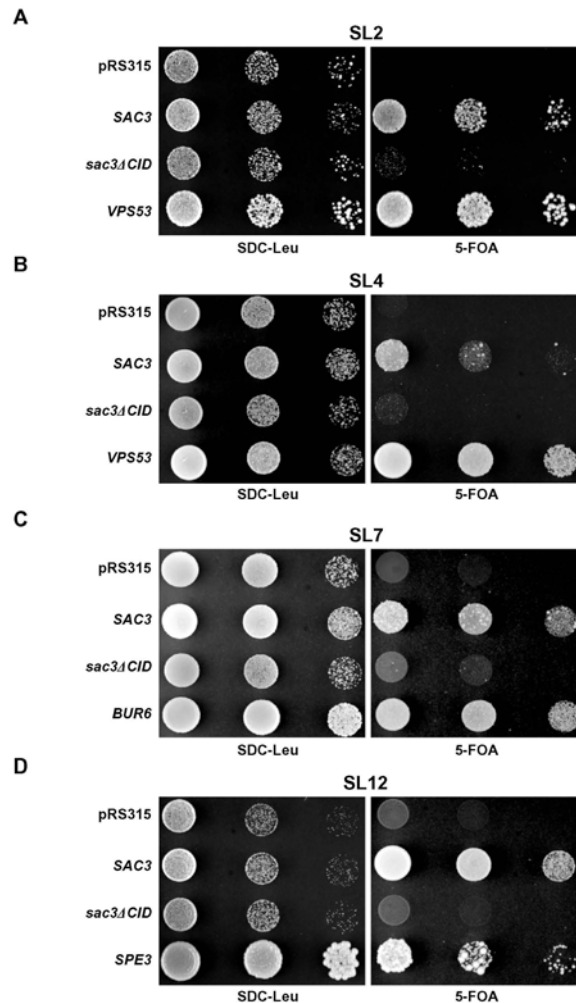
To this end, I transformed 50 *sac3Δ* SL mutants with an allele of *SAC3* lacking the CID motif (*sac3ΔCID*) and checked for viability of these strains on 5-FOA plates after shuffling out the wild-type copy of *SAC3* (Fig. 7).



**Figure 7 Strategy of the screen for genetic interaction with *Sac3<sup>CID</sup>***

SL mutants of the initial *sac3Δ* SL screen (see above and Materials and Methods) which harbour a genomic mutation (designated as *mut1*) leading to an SL phenotype with *sac3Δ* were examined. All mutants carry a *URA3* plasmid with a wild-type copy of *SAC3* and were transformed with a *LEU2* plasmid carrying *sac3ΔCID* (see text). If the SL mutant is complemented by *sac3ΔCID*, the wild-type *SAC3* plasmid can be lost and cells grow on 5-FOA. Mutants that could not be complemented by *sac3ΔCID* were selected for further analysis.

Of the 50 strains tested, ten were synthetically lethal with *sac3ΔCID* while growth could be restored by transformation with *SAC3*, indicating that they depend on a functional CID for complementation. These SL mutants were selected for further analysis by transformation with a yeast genomic library to clone the genes that are genetically interacting with *SAC3<sup>CID</sup>*. From four of these ten strains, complementing plasmids could be isolated and single genes were identified that could rescue growth of these SL candidates on 5-FOA plates (Fig. 8).



**Figure 8 Complementation of *sac3Δ* SL mutants by *VPS53*, *BUR6* and *SPE3*.**

*sac3Δ* SL mutants were transformed with the empty vector pRS315 or pRS315 carrying the genes of *SAC3*, *sac3ΔCID* and (A+B) *VPS53*, (C) *BUR6* or (D) *SPE3*. The same number of transformed cells was spotted to SDC-Leu plates (as loading control) and 5-FOA plates in tenfold serial dilutions and incubated for 4 days at 30°C. No growth on 5-FOA indicates synthetic lethality.

The gene *VPS53*, found to complement the mutants SL2 and SL4 (Fig. 8A and 8B), codes for a protein which was originally identified as a component of the GARP (Golgi-associated retrograde protein) complex. This complex is required for retrograde transport of proteins from endosomes to the Golgi apparatus by interaction with Tlg1, a Golgi-standing SNARE mediating membrane fusion of vesicles with the late Golgi membrane (Liewen et al., 2005; Siniosoglou and Pelham, 2002). The link to vesicular trafficking documented here is difficult to interpret and could be due to an indirect effect of the function of *SAC3<sup>CID</sup>* in transcription-coupled mRNA export.

*BUR6*, the gene complementing the *sac3Δ* SL mutant SL7 (Fig. 8C), encodes a subunit of the yeast homologue of the NC2 transcriptional regulator complex in

mammals (Goppelt and Meisterernst, 1996). In yeast, Bur6 forms a heterodimer with the second subunit of yeast NC2, Ncb2, and interacts with and selectively inhibits TATA-binding protein (TBP) complexes leading to a negative regulation of transcription (Geisberg et al., 2001; Goppelt et al., 1996). *BUR6* has previously been found in an SL screen with an allele of the DEAD-box RNA helicase *DBP5*, suggesting a connection of the transcription factor *BUR6* with mRNA export (Estruch and Cole, 2003).

The SL phenotype of mutant SL12 could be complemented by *SPE3*, the gene coding for spermidine synthase (Fig. 8D). This enzyme of 293 amino acids catalyzes the reaction of putrescine and decarboxylated S-adenosylmethionine to spermidine (Hamasaki-Katagiri et al., 1997). Spermidine is a small and basic molecule required for the hypusination of eIF5A, the eukaryotic translation initiation factor 5A (Chattopadhyay et al., 2008; Chattopadhyay et al., 2003), and was also implied in nuclear processes like transcriptional repression of sporulation-specific genes (Friesen et al., 1998) and global chromatin reorganisation by interaction with nucleosomes (Morgan et al., 1987; reviewed in Childs et al., 2003). In a recent study, administration of spermidine to yeast cells was furthermore found to change the acetylation status of histone H3 by inhibition of histone acetyltransferases (Eisenberg et al., 2009). Due to the interesting roles of Spe3 in nuclear processes, I sought to characterize the mutant SL12 and the genetic interaction of *SAC3* and *SPE3* further.

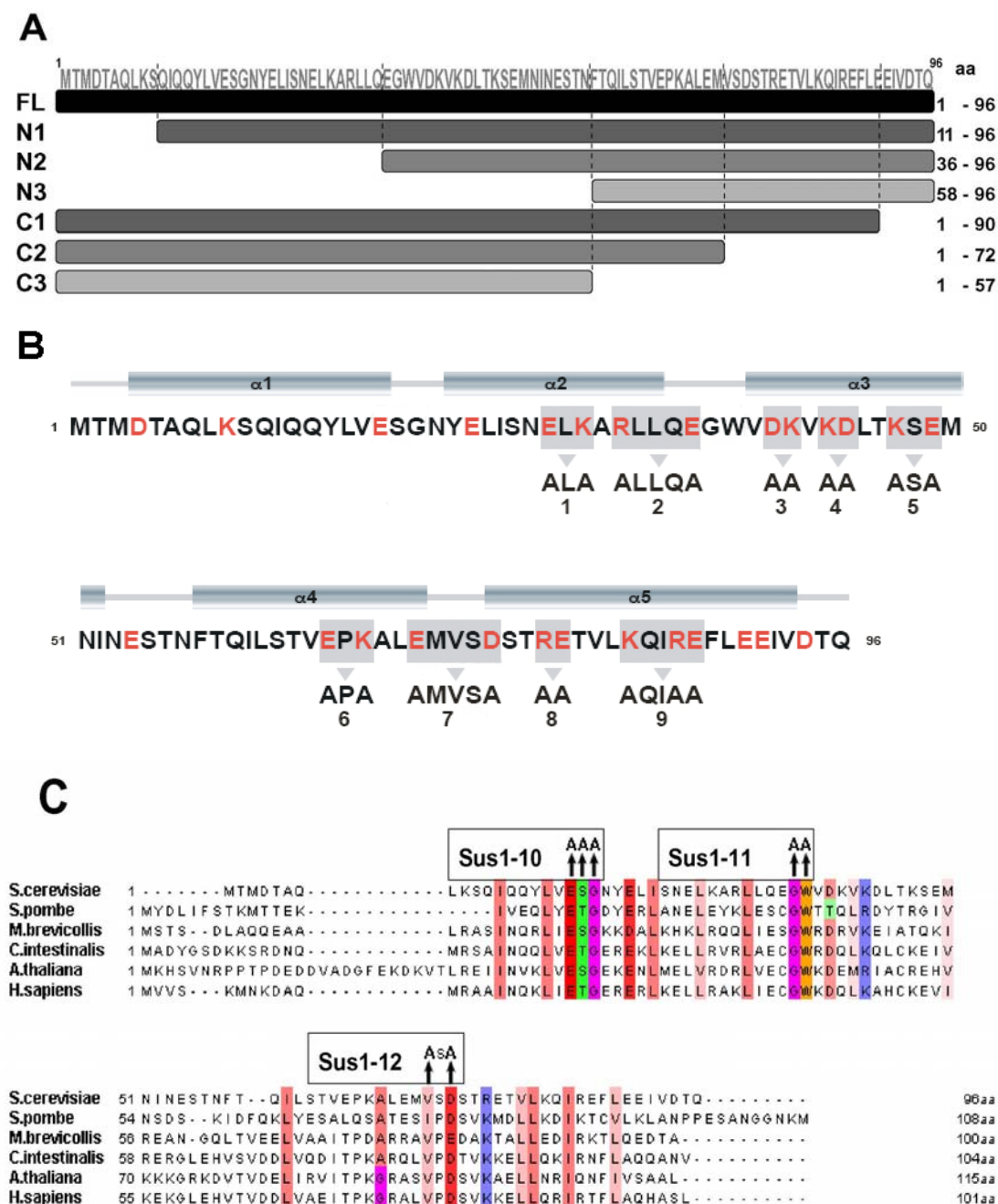
SL12 was found to contain a nonsense mutation in the genomic *SPE3* locus (Q181-stop), leading to a C-terminal truncation of 113 amino acids, presumably disturbing the function of Spe3. To test the dependence on spermidine synthase activity for complementation of SL12, I transformed the SL mutant with the allele *spe3D168A*. This allele contains a mutation in the predicted catalytic center of the yeast spermidine synthase, which should inactivate the enzymatic activity of Spe3 (Ikeguchi et al., 2006; Korolev et al., 2002). When the allele was tested in SL12, it could not complement the SL phenotype (data not shown), suggesting that the spermidine synthase activity of Spe3 is responsible for complementation. However, the analysis of a distinct *spe3Δsac3Δ* double knockout strain which was subsequently generated in the same genetic background as the original SL screening strain (see Table III, Materials and Methods) revealed that the distinct combination of the two deletions remained viable after losing the *URA3* plasmid carrying wild-type *SAC3* (data not shown). A possible explanation for the discrepancy between SL12 and

*spe3Δsac3Δ* could be an additional mutation in the SL mutant which is not present in the double deletion. Nevertheless, the effect of *SPE3* on the described *sac3Δ* SL candidate still represents an indication that Spe3 might be linked to transcription-coupled mRNA export. Due to the genetic complexity of this interaction, which could not be further narrowed down, I did not continue with the analysis of a possible functional link between *SAC3<sup>CID</sup>* and *SPE3*.

Taken together, this genetic approach yielded novel genetic interactions of *SAC3<sup>CID</sup>* stressing the connection of this motif with transcriptional regulation. While the interaction between *SAC3<sup>CID</sup>* and *VPS53* is difficult to interpret, the genetic relations of *SAC3<sup>CID</sup>* with *BUR6* and *SPE3* indicate a functional link of these factors to transcription-coupled mRNA export and could be interesting to follow in future studies.

## **2.2 Comprehensive mutagenesis of Sus1 to identify crucial residues for the interaction with TREX-2 or SAGA**

The protein Sus1 functions in transcription as well as mRNA export through its interaction with the transcriptional coactivator SAGA and the mRNA export complex TREX-2 (Rodriguez-Navarro et al., 2004). In order to better understand how Sus1 binds to Sac3<sup>CID</sup> within TREX-2 and whether this interaction is similar or different to how Sus1 interacts with SAGA, I performed a mutagenesis approach with Sus1. As a goal, I wanted to obtain information on how Sus1 can interact with SAGA and TREX-2 and whether binding to one or the other complex can be selectively disrupted by specific mutations of Sus1. To identify regions or residues of Sus1 important for the association with SAGA or TREX-2, I used three different mutagenesis strategies: truncations from the N- and the C-terminus (Fig. 9A), mutagenesis of clusters of charged residues to alanine (Fig. 9B) and mutagenesis of residues in putative turn regions of the Sus1 secondary structure to alanine (Fig. 9B+9C).



**Figure 9 Overview of the generated truncations and site-directed mutations of Sus1.**

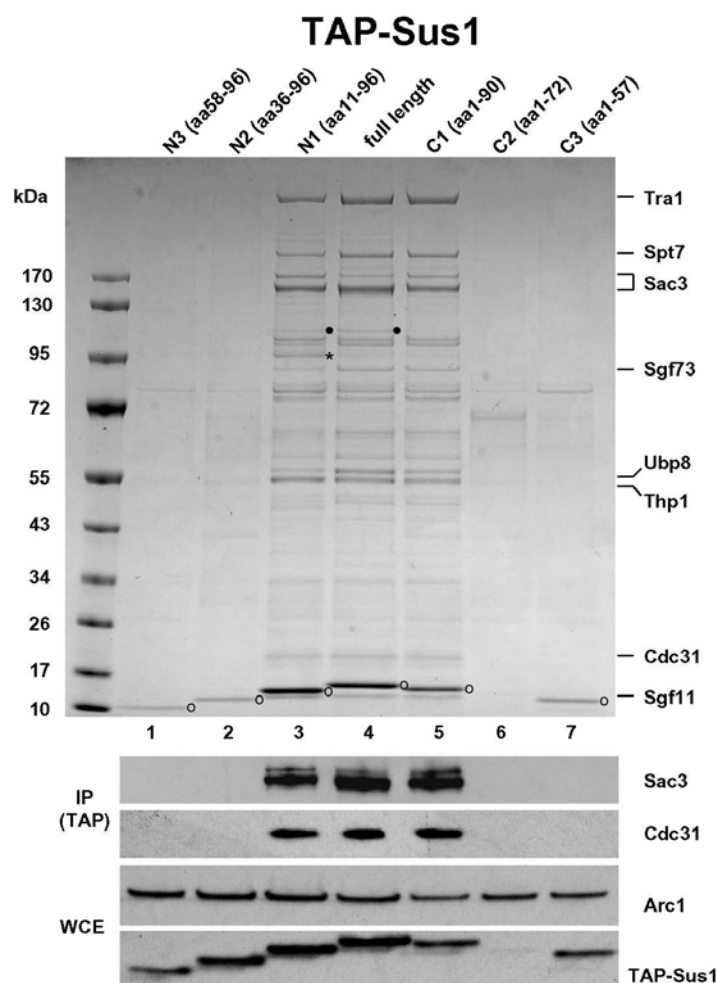
(A) Schematic overview of N- and C-terminal truncations. Shown on top is the amino acid sequence, bars represent full-length Sus1 (FL) or N- and C-terminal truncations (N1-N3, C1-C3). Dashed lines show borders of the truncations, numbers on the right indicate amino acids comprised by the truncation constructs. (B) Schematic overview of charge-to-alanine mutations. In the protein sequence of Sus1, clusters of charged amino acids selected for mutation are shaded in grey and individual charged residues are highlighted in red. Resulting mutated residues are indicated below the protein sequence. Charge-to-alanine mutants are numbered according to their position in Sus1 (1-9). A secondary structure prediction of Sus1 is shown above the protein sequence of Sus1. (C) Schematic overview of mutations of conserved residues. Identical and similar residues in the primary sequence of Sus1 in different species are highlighted (protein sequence alignment of Sus1 from representative species including fungi, protozoa, plants and metazoans). Arrows indicate the individual residues mutated to alanine and are shown in boxes with the corresponding allele number (Sus1-10, Sus1-11 and Sus1-12).

The different strategies shown in Fig. 9 were used to generate a series of mutants of Sus1. All mutants were subsequently tested for their interaction with the SAGA or TREX-2 complex by affinity purification. The study of these mutants should reveal if there are residues or regions that affect binding of Sus1 to TREX-2 or SAGA differently.

### **2.3 The less conserved N- and C-terminal residues of Sus1 are not involved in the association with TREX-2 or SAGA**

Initially, I sought to determine which parts of the 96-residue protein Sus1 are necessary for the interaction with SAGA or TREX-2. To test for the individual contribution of N- or C-terminal portions of Sus1 to binding to the partner complexes, I progressively truncated Sus1 from the N-terminus as well as from the C-terminus (Fig. 9A) and tested these constructs for interaction with SAGA and TREX-2.





**Figure 10 Truncation of less conserved N-or C-terminal residues of Sus1 leave the association with SAGA and TREX-2 largely unaffected**

Affinity purifications from *sus1Δ* cells expressing plasmid-borne TAP-tagged full length or indicated N- or C-terminal truncation constructs of Sus1. Protein constructs were purified according to the TAP method (see Materials and Methods) and final eluates were analyzed by SDS-PAGE (4-12% gradient gel, MOPS buffer) and Coomassie staining. Truncation constructs are numbered according to Figure 9A, borders of truncations are shown in parentheses. Indicated Coomassie-stained bands were determined by mass spectrometry. The asterisk labels a contaminant of the purification, Crn1, and filled circles indicate a degradation product of Sac3. Open circles show the position of Sus1 truncation constructs. Full length Sus1 was purified and shown for comparison. Purified protein eluates (TAP) were analyzed by Western blotting using  $\alpha$ -Sac3 and  $\alpha$ -Cdc31 antibodies. Expression levels in whole cell extracts (WCE) were analyzed by Western blotting followed by detection of the protein A tag and compared to Arc1 (yeast cytosolic marker protein).

When Sus1 is purified by the TAP method, it efficiently co-enriches both SAGA and TREX-2 complex subunits (Rodriguez-Navarro et al., 2004; Fig. 10, lane 4). The deletion of the 10 N-terminal residues (Sus1-N1; aa11-96) or 6 C-terminal residues (Sus1-C1, aa1-90) of Sus1 did not cause major changes in the pattern of co-purified proteins, indicating that these constructs were still stably associated with SAGA and TREX-2 (Fig. 10, lane 3 and lane 5). However, more extensive truncations of Sus1

(Sus1-N3, Sus1-N2 and Sus1-C3; see Fig. 9A for a schematic of the truncations) caused a concomitant decrease of co-purified SAGA and TREX-2 proteins (Fig. 10, lane 1, lane 2 and lane 7). In the case of Sus1-C2, the truncation of Sus1 led to a marked decrease in the yield of the purification and decreased expression levels of the truncation construct (Fig. 10, lane 6).

Taken together, the truncation of the less conserved 10 N-terminal amino acids or 6 amino acids from the C-terminus of Sus1 leaves the interaction with SAGA and TREX-2 largely intact, while more extensive truncations that affect conserved core residues of Sus1 lead to a concomitant decrease of association with SAGA and TREX-2 subunits or decreased expression levels of the mutant proteins.

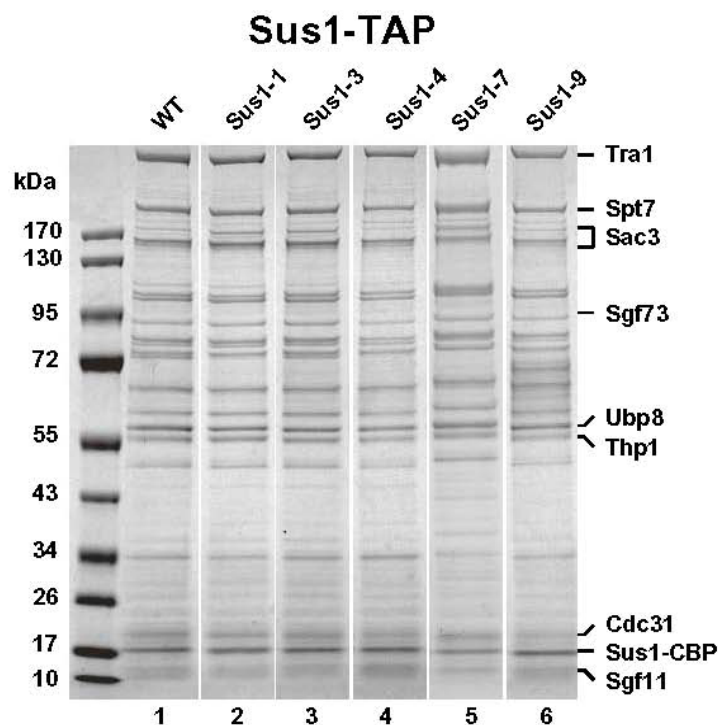
## **2.4 Mutations of charged amino acid clusters to alanine do not impair the functions of Sus1**

Since N- and C-terminal truncations of Sus1 did not lead to a selective dissociation of Sus1 from TREX-2 or SAGA, I sought to employ site-specific mutagenesis to generate further mutants of Sus1. David Botstein and colleagues developed a so-called “clustered charge-to-alanine” mutagenesis protocol to mutate crucial amino acid clusters on the protein surface of actin that impair the function of the protein (*ACT1*; Wertman et al., 1992). The rationale of this mutagenesis was to mutate clusters of charged residues in the primary sequence to alanine. Charged clusters tend to be on the surface of proteins and could be involved in mediating protein-protein interactions. The mutations of these residues to alanine should abolish interactions that are mediated by the charge of the residues without disturbing the overall structural fold of the protein, as was shown for example for the human tissue plasminogen activator (tPA; Bennett et al., 1991).

This described approach was adopted for Sus1 to mutate charged residues that could be involved in the interaction with SAGA or TREX-2, respectively.

According to the “clustered charge-to-alanine” strategy, I selected clusters of charged residues in the protein sequence of Sus1 (Wertman et al., 1992): these were identified by looking at segments of five residues and defining them as clusters if any given segment contained two or more charged amino acids (aspartate, glutamate, lysine or

arginine). The charged residues of an identified cluster were consequently mutated to alanine and resulting mutants were analyzed experimentally. Applying this approach, I could scan the Sus1 sequence for charged residues that are potentially involved in protein-protein interactions. Overall, 9 charged clusters were delineated and charged residues were mutated to alanine (Fig. 9B). A cluster of charged residues at the very C-terminus was not mutated due to the dispensability of this region for interaction with TREX-2 and SAGA (see Fig. 10). Representative affinity purifications of five charge-to-alanine mutants are shown in Fig. 11.

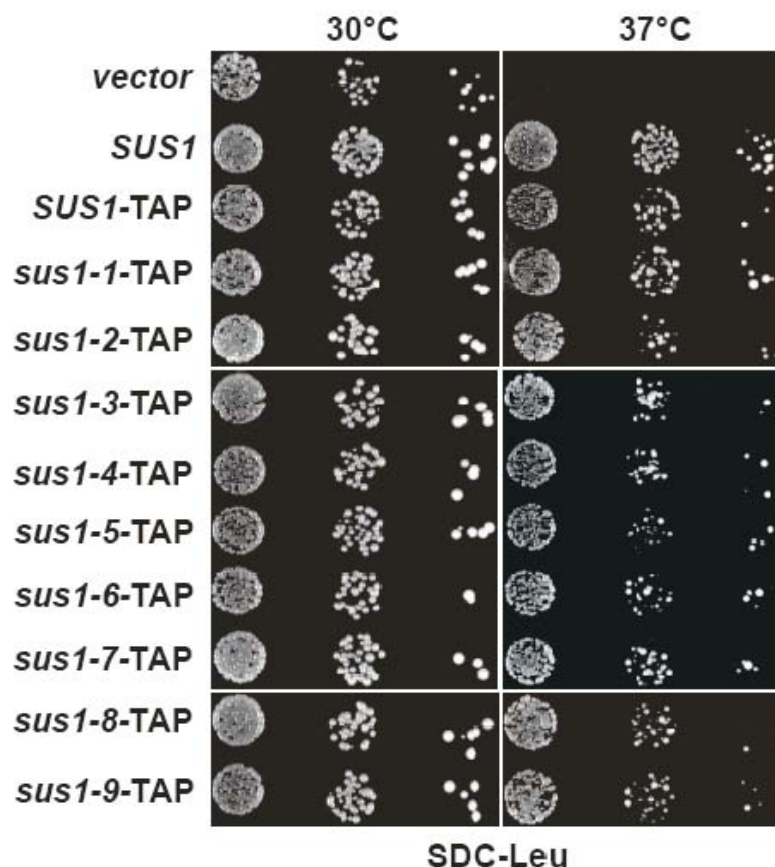


**Figure 11 Charge-to-alanine mutations of Sus1 do not significantly affect SAGA or TREX-2 interaction**

Affinity purifications from *sus1Δ* cells expressing plasmid-bourne TAP-tagged wild-type (WT) Sus1 or indicated mutants. Proteins were purified according to the TAP method (see Materials and Methods) and final eluates were analyzed by SDS-PAGE (4-12% gradient gel, MOPS buffer) and Coomassie staining. Mutations are numbered according to Fig. 9B. Protein bands are indicated according to the molecular mass of proteins reproducibly appearing in Sus1-TAP purifications and previously identified by mass spectrometry (Köhler et al., 2008).

Unexpectedly, all nine charge-to-alanine Sus1-TAP mutants co-purified similar amounts of TREX-2 (Sac3, Thp1 and Cdc31) and SAGA components (*e.g.* Tra1 and Spt7) as compared to wild-type Sus1-TAP (Fig. 11 and data not shown). These results indicate that the selected charged residues do not play a major role in the association of Sus1 with SAGA and TREX-2.

Next, I wanted to find out whether the charge-to-alanine mutations impair cell growth at elevated temperatures. To this end, *sus1Δ* cells shown to exhibit a slight growth defect at 30°C and a temperature-sensitive (ts) phenotype at 37°C (Rodriguez-Navarro et al., 2004), were transformed with plasmids expressing charge-to-alanine mutants 1 to 9 and growth was analyzed at 30°C and 37°C.



**Figure 12 Growth analysis of charge-to-alanine *sus1* mutants in *sus1Δ* cells**

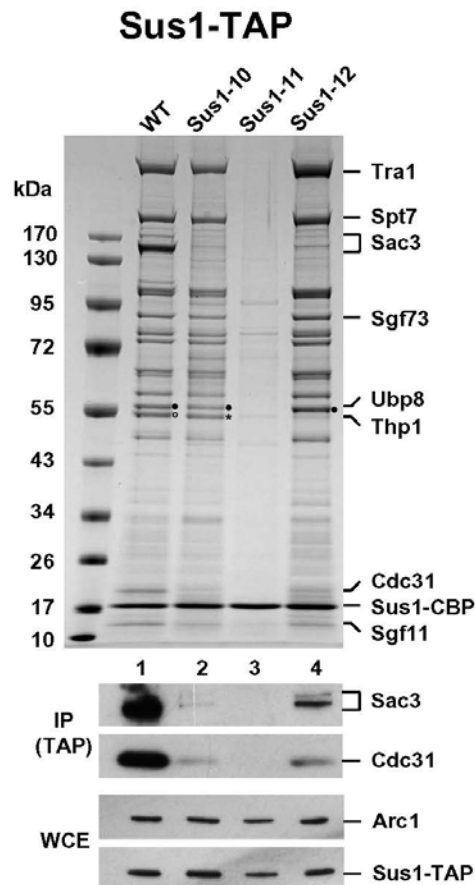
*sus1Δ* cells were transformed with the empty vector pRS315 or pRS315 carrying the indicated *SUS1* constructs. The same number of transformed cells was spotted to SDC-Leu plates in tenfold serial dilutions and incubated for 2 days at 30°C or 37°C. *sus1Δ* transformed with empty vector, untagged *SUS1* and wild-type *SUS1*-TAP are shown for comparison.

Consistent with the biochemical data, no apparent growth defect at 37°C was observed when *sus1Δ* cells were expressing charge-to-alanine mutants 1-9 (Fig. 12). Moreover, the TAP tag attached to Sus1 does not interfere with the function of the protein. In conclusion, site-directed mutagenesis of Sus1 covering nearly all charged residues in clusters did not lead to an impairment of the functions of Sus1. Alternatively, the mutation of a combination of charged clusters may generate a phenotype not seen with the mutations of single clusters.

Taken together, the interaction of Sus1 with its binding partners appears not to be disturbed by mutation of the selected charged clusters. Thus, we were wondering which types of interaction could be involved in the interaction of Sus1 with its partners.

## **2.5 Conserved residues in predicted secondary structure turn regions of Sus1 differentially regulate the interaction with TREX-2 or SAGA**

Since neither short N- and C-terminal truncations nor charge-to-alanine scan mutagenesis impaired the interaction of Sus1 with TREX-2 and SAGA, I generated site-directed mutations in putative turn regions between  $\alpha$ -helices of Sus1 predicted by sequence analysis (Fig. 9B; Sus1-10, Sus1-11 and Sus1-12). Each of these mutations involves residues that are often found in flexible turn regions, as glycine or aspartate. Furthermore, the residues that were selected for mutagenesis are evolutionary conserved (Fig. 9C). These Sus1 mutants were tested for interaction with SAGA and TREX-2 factors.



**Figure 13 Mutations of conserved residues in predicted turn regions of Sus1 differentially affect the association with SAGA or TREX-2**

Affinity purification of genomically expressed TAP-tagged wild-type Sus1 (WT), Sus1-10, Sus1-11 or Sus1-12. Proteins were purified according to the TAP method (see Materials and Methods) and final eluates were analyzed by SDS-PAGE (4-12% gradient gel, MOPS buffer) and Coomassie staining. Bands are indicated according to the molecular mass of proteins reproducibly appearing in Sus1-TAP purifications and previously identified by mass spectrometry (Köhler et al., 2008). Filled circles indicate Ubp8 and open circles indicate Thp1. The asterisk indicates a contaminant, eEF-1 $\alpha$ , running slightly below Thp1. In this experiment, Ubp8, Thp1 and eEF-1 $\alpha$  have been identified by mass spectrometry. Purified protein eluates (TAP) were analyzed by Western blotting using  $\alpha$ -Sac3 and  $\alpha$ -Cdc31 antibodies. Expression levels of Sus1-TAP in whole cell extracts (WCE) were analyzed by Western blotting followed by protA detection (protein A is part of the TAP tag) and compared to Arc1 (yeast cytosolic marker protein).

Strikingly, the affinity purifications of TAP-tagged Sus1-10, Sus1-11 and Sus1-12 differed substantially from wild-type Sus1. Sus1-10-TAP and Sus1-12-TAP co-purified typical SAGA subunits (*e.g.* Tra1 and Spt7), including the DUB module components Sgf73, Ubp8 and Sgf11 while the co-purification of TREX-2 factors (Sac3, Thp1 and Cdc31) was significantly reduced. Western blot detection of Sac3 and Cdc31 revealed that the loss of TREX-2 factors was more pronounced for Sus1-10 than for Sus1-12 (Fig. 13). These data suggest that the association of Sus1-10 and

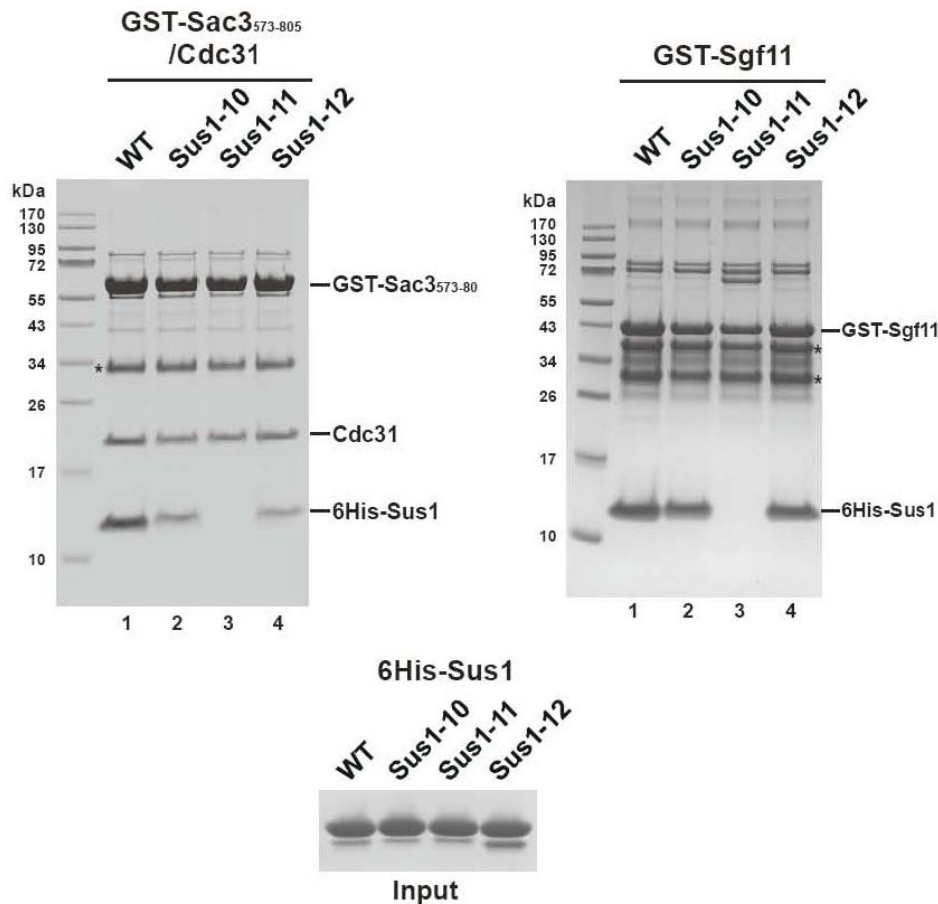
Sus1-12 with the DUB module (Sus1-Sgf11-Sgf73-Ubp8) inside the SAGA complex remains largely unaffected while the interaction with TREX-2 was perturbed.

Unlike Sus1-10 and Sus1-12, TAP-tagged Sus1-11 exhibited a striking loss of both SAGA and TREX-2 factors, which is apparent both on the Coomassie-blue stained gel and immunoblots against Sac3 and Cdc31 (Fig. 13). The reduction or absence of co-purified proteins from one or the other complex was not due to lower protein levels of mutant Sus1 versions as these showed similar expression levels in yeast with a marginal reduction for Sus1-11 (Fig. 13).

These results suggest that for Sus1-11, biochemical interaction with SAGA and TREX-2 was disrupted while mutations in Sus1-10 and Sus1-12 selectively disturb binding to TREX-2. Decrease or loss of Sus1 interaction with SAGA or TREX-2 might have been due to reduced or abolished interaction of the mutant proteins with the respective binding partners Sgf11 (Köhler et al., 2006; Köhler et al., 2008) and the CID motif of Sac3 (Fischer et al., 2004).

## **2.6 Mutants of Sus1 bind to Sac3 and Sgf11 with different affinities *in vitro***

After three mutants of Sus1 could be identified that show impaired binding to TREX-2 (Sus1-10 and Sus1-12) or TREX-2 and SAGA (Sus1-11), we sought to confirm that the impaired association of the Sus1 mutants with TREX-2 or SAGA was mainly caused by a reduced affinity between Sus1 and Sac3 or Sus1 and Sgf11, respectively. To this end, recombinant wild-type or mutant Sus1 proteins were first tested for their ability to bind to a Sac3 (573-805)-Cdc31 heterodimer. The C-terminal fragment of Sac3 used in this experiment contains the binding sites for Sus1 and Cdc31 (Fischer et al., 2004). Heterodimer formation between Sus1 mutants and Sgf11, the binding partner of Sus1 in SAGA (Köhler et al., 2006; Köhler et al., 2008), was also tested in this *in vitro* experiment.



**Figure 14 Mutants of Sus1 bind to Sac3 and Sgf11 with different affinities *in vitro***

Purified GST-Sac3 (573-805)-Cdc31 complex or GST-Sgf11 expressed in *E. coli* were bound to GSH agarose and incubated with recombinant wild-type (WT) or mutant 6xHis-Sus1 (input). Proteins were eluted from the beads with GSH and analyzed by SDS-PAGE (12% gel, MES buffer) and Coomassie staining. Asterisks indicate Sac3 and Sgf11 degradation products (identified by mass spectrometry). This experiment was done in collaboration with A. Köhler and M. Schneider (Hurt laboratory).

Wild-type Sus1 bound to the Sac3 (573-805)-Cdc31 heterodimer very efficiently, while the binding was reduced with Sus1-10 and Sus1-12 and largely abolished for the Sus1-11 mutant. The binding of Sus1-10 and Sus1-12 to Sgf11, on the other hand, was largely unaffected, whereas Sus1-11 failed to interact with Sgf11 (Fig. 14). Misfolding of mutant Sus1 proteins as a reason for disturbed Sus1-ligand interactions appears improbable because the CD spectra of wild-type and mutant Sus1 were determined and found to be virtually indistinguishable (prominent negative ellipticities at 220 nm consistent with the presence of an  $\alpha$ -helical conformation; personal communication with D. Jani, group of M. Stewart, MRC-LMB, Cambridge). We observed that the dissociation of Sus1-10 and Sus1-12 from Sac3 (573-805)-Cdc31 in this *in vitro* assay is not as pronounced as would have been expected from

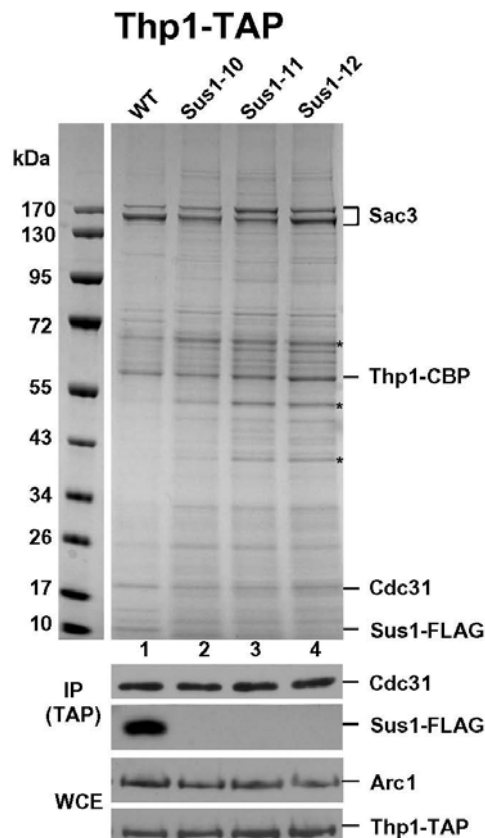


the yeast affinity purifications. This might be due to an excess of ligand protein in this assay.

Taken together, the *in vitro* reconstitution of mutants with their ligands showed similar interaction defects as the *in vivo* affinity purification experiment. Sus1-11 failed to interact with both Sgf11 (the binding partner in SAGA) and Sac3 (the binding partner in TREX-2) while the interaction deficiency of Sus1-10 and Sus1-12 only affects the interaction with the binding partner in TREX-2.

## **2.7 Sus1 dissociation from TREX-2 does not affect the association of Sac3, Thp1 and Cdc31 with each other**

The co-enrichment of TREX-2 components with the alleles Sus1-10, -11 and -12 is significantly reduced in yeast affinity purifications (Fig. 13). This was shown to be caused by a reduced affinity of these mutants to the binding partner of Sus1 in TREX-2, Sac3 (Fig. 14). As Sus1 was previously shown to bind to the same C-terminal region of Sac3 as Cdc31, the Sac3<sup>CID</sup> motif (Fischer et al., 2004), it seemed possible that a disturbed Sus1-Sac3<sup>CID</sup> interaction would influence binding of Cdc31 to this motif. To characterize the effects of the different *sus1* alleles on TREX-2 subunit composition, TREX-2 was purified over TAP-tagged Thp1 and the amount of co-purified FLAG-tagged wild-type or mutant Sus1 protein was determined by Western analysis.



**Figure 15 Sac3, Thp1 and Cdc31 remain associated in the absence of Sus1**

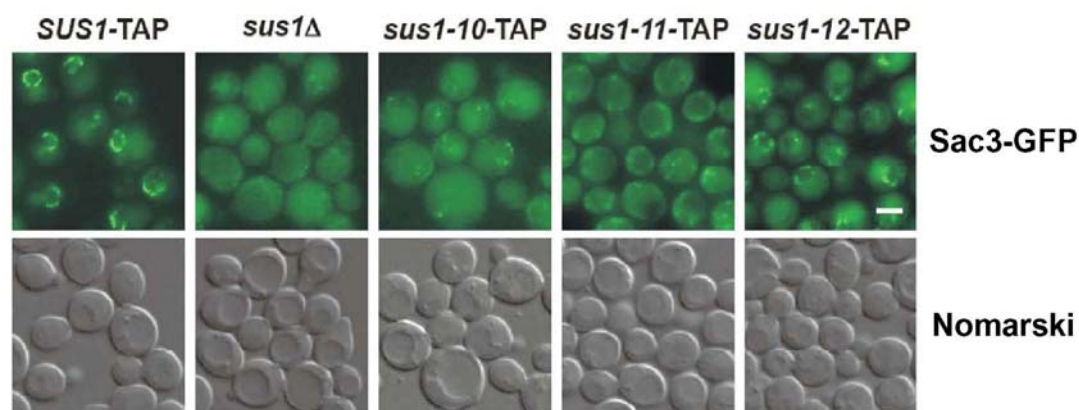
Affinity purification of Thp1-TAP from strains genomically expressing FLAG-tagged wild-type Sus1, Sus1-10, Sus1-11 or Sus1-12. Proteins were purified according to the TAP method (see Materials and Methods) and final eluates were analyzed by SDS-PAGE (4-12% gradient gel, MOPS buffer) and Coomassie staining. Indicated Coomassie stained protein bands were identified by mass spectrometry. Asterisks label unspecific contaminants of the purification (from top to bottom: keratin, Eno2 and Tdh3). Purified protein eluates (TAP) were analyzed by Western blotting using  $\alpha$ -FLAG and  $\alpha$ -Cdc31 antibodies. Expression levels of Thp1-TAP in whole cell extracts (WCE) were analyzed by Western blotting followed by protA detection (protein A is part of the TAP tag) and compared to Arc1 (yeast cytosolic marker protein).

Thp1-TAP co-purifies the TREX-2 subunits Sac3, Cdc31 and Sus1 when expressed in a wild-type background (Fig. 15, lane 1). When Thp1-TAP was purified from cells expressing the FLAG-tagged mutants Sus1-10, Sus1-11 or Sus1-12, these were not detectable (Fig. 15, lanes 2-4). However, Sac3 and Cdc31 could be co-purified with Thp1-TAP independent of the association with Sus1. Disruption of Sus1 binding to TREX-2 did not have an influence on the stability of Thp1-TAP as expression levels of Thp1-TAP were similar in all *sus1* backgrounds.

In summary, the absence of Sus1 from TREX-2 does not affect the association of the other TREX-2 subunits Sac3, Thp1 and Cdc31 with each other.

## 2.8 Association of Sus1 with TREX-2 is required for NPC localization and mRNA export

Sus1 was previously shown to be required for targeting of Sac3-GFP and Thp1-GFP to the nuclear periphery (Köhler et al., 2008). Sac3-GFP shows a typical nuclear rim stain when expressed in a wild-type *SUS1* background while the fluorescent signal is shifted to the cytoplasm and the nucleoplasm in *sus1Δ* cells. To test the impact of mutant *sus1* alleles on NPC targeting of TREX-2, I examined the localization of Sac3-GFP in *sus1-10-TAP*, *sus1-11-TAP* and *sus1-12-TAP* cells.



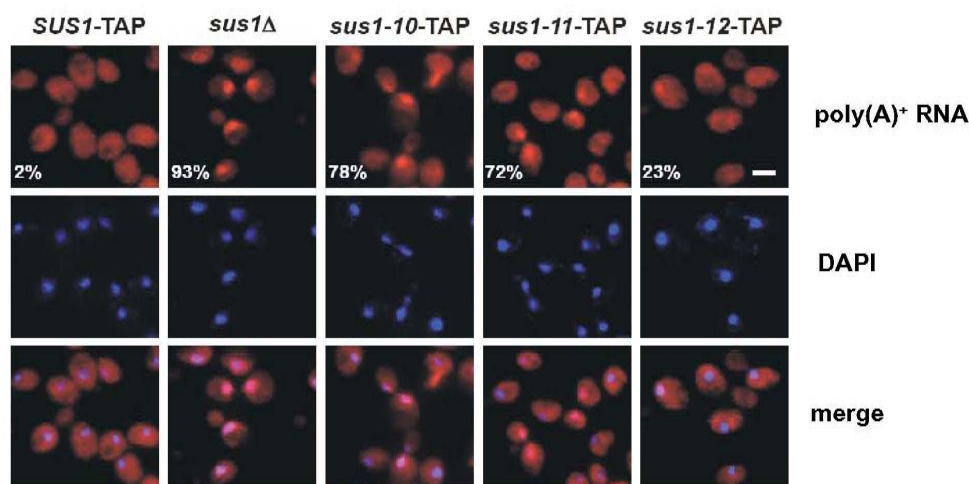
**Figure 16 Uncoupling of Sus1 from TREX-2 causes mislocalization of Sac3-GFP**

Subcellular localization of Sac3-GFP in the indicated wild-type *SUS1*-TAP and *sus1*-TAP mutant strains was analyzed by Nomarski imaging and fluorescence microscopy. Cells were grown at 30°C before analysis. *SUS1*-TAP and *sus1Δ* cells are shown for comparison. Scale bar, 2μm.

All three *sus1* alleles tested lead to an altered pattern of Sac3-GFP staining, confirming the requirement of Sus1 binding to Sac3 for correct localization of TREX-2 to nuclear pore complexes (Fig. 16). *sus1-11-TAP* led to the most pronounced mislocalization of Sac3-GFP, comparable to the situation in *sus1Δ* cells. Targeting of TREX-2 to the nuclear pore was previously shown to be independent of Sus1 interaction with SAGA as deletion of *UBP8* (leading to dissociation of Sus1 from SAGA) leaves Sac3-GFP localization to NPCs intact (Köhler et al., 2008). Thus, mislocalization of Sac3-GFP in *sus1-11-TAP* cells should be solely attributed to defects of the mutant in TREX-2. *sus1-10-TAP* caused a slightly stronger defect of Sac3-GFP targeting to the nuclear periphery than *sus1-12-TAP*, consistent with a lower residual interaction with TREX-2 seen in Sus1-TAP experiments (Fig. 13).

In summary, defective association of Sus1 with Sac3<sup>CID</sup> and the TREX-2 complex leads to impaired targeting of Sac3-GFP to the nuclear periphery. The mislocalization of Sac3-GFP in *sus1-10*-TAP, *sus1-11*-TAP and *sus1-12*-TAP cells (Fig. 16), taken together with the TREX-2 association defects of the mutant *sus1* alleles seen in Fig. 15, suggests a requirement for correct assembly of Sus1 into TREX-2 for correct NPC targeting of the whole complex.

The incorrect localization of the TREX-2 complex to NPCs was previously observed to be connected to an mRNA export defect (Köhler et al., 2008). To test the influence of Sus1-10, Sus1-11 and Sus1-12 on nuclear mRNA export, the localization of poly(A)<sup>+</sup> RNA was analyzed in the respective strains.



**Figure 17 Impaired association of Sus1 with TREX-2 leads to mRNA export defects**

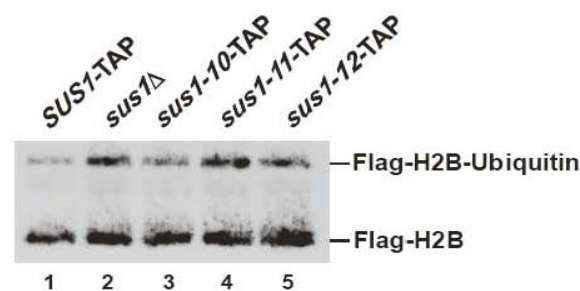
Analysis of nuclear poly(A)<sup>+</sup> RNA export in the indicated wild-type *SUS1*-TAP and *sus1*-TAP mutant strains. Cells growing in mid-log phase were shifted to 37°C for 2 hours and poly(A)<sup>+</sup>RNA was detected by fluorescent *in situ* hybridization with Cy3-labeled oligo (dT) probes. DNA was visualized using 4,6-diamidino-2-phenylindole (DAPI). Percentages indicate the ratio of cells (~200 cells counted per strain) that exhibit a clear mRNA export defect. *SUS1*-TAP and *sus1*Δ are shown for comparison. Scale bar, 2μm.

In wild-type cells, mRNA is rapidly exported from the nucleus. When assayed by fluorescent *in situ* hybridization for poly(A)<sup>+</sup> RNA, mRNA is detected mainly in the cytoplasm with a certain degree of nuclear exclusion (Fig. 17, *SUS1*-TAP). When mRNA export is impaired, the fluorescent signal accumulates in the nucleus and decreases in the cytoplasm. As shown in Fig. 17, all mutants analyzed exhibited poly(A)<sup>+</sup> RNA retention in the nucleus after a 2 hour shift to the restrictive temperature, however to different extents. *sus1-10*-TAP and *sus1-11*-TAP cells displayed a strong export defect while the mutations in *sus1-12*-TAP led to a milder

impairment in mRNA export with less cells affected. These observations are consistent with results obtained with the *sus1-12-TAP* allele in previous experiments. Taken together, the mRNA export defect caused by mutants of *SUS1* can be attributed to their impaired assembly into TREX-2 and consequent mislocalization of the complex, as *Sus1-10-TAP* and *Sus1-12-TAP* were previously shown to be selectively uncoupled from TREX-2 and to cause impaired NPC targeting of the complex.

## 2.9 Mutants of *Sus1* influence the deubiquitination of histone H2B

The co-enrichment of factors from the DUB module (*Sus1-Sgf11-Ubp8-Sgf73*) of SAGA with *Sus1-10-TAP* and *Sus1-12-TAP* in affinity purifications (Fig. 13) indicated a correct structural assembly of the module. However, it was still possible that the enzymatic activity of Ubp8 is disturbed. It was recently shown that a deletion of *SUS1* leads to higher levels of global Ub-H2B due to dissociation of Ubp8 from the DUB module and SAGA (Köhler et al., 2008). This dissociation was furthermore shown to lead to enzymatic inactivation of the deubiquitinating protease Ubp8 (Köhler et al., 2006; Lee et al., 2005). To test for intact Ubp8 function, global levels of ubiquitinated H2B (Ub-H2B) were assayed in mutant *sus1* backgrounds.



**Figure 18 Deubiquitination of histone H2B is slightly impaired in *sus1-10-TAP* and *sus1-12-TAP***

Analysis of global histone H2B ubiquitin levels in strains expressing mutant *sus1* alleles. Anti-FLAG-H2B immunoprecipitates from mutant *sus1-TAP* cells were analyzed by SDS-PAGE and Western blotting. Unmodified FLAG-H2B and ubiquitinated FLAG-H2B were detected using an  $\alpha$ -FLAG antibody. Wild-type *SUS1-TAP* and *sus1Δ* are shown for comparison. This experiment was performed by A. Köhler and M. Schneider (Hurt laboratory).

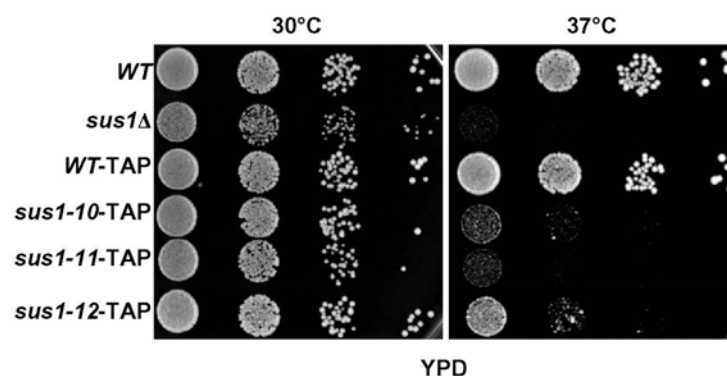
As expected, the complete dissociation of *Sus1-11-TAP* from the DUB module led to a defective deubiquitination of Ub-H2B and levels of FLAG-H2B-Ubiquitin

comparable to *sus1Δ* (Fig. 18). Although biochemical and functional assays performed with Sus1-10-TAP and Sus1-12-TAP indicated a correct assembly into the DUB module, levels of Ub-H2B slightly higher than the wild-type reference point to a reduction of Ubp8 activity.

Taken together, the levels of Ub-H2B seen in a *sus1-11*-TAP background are consistent with its disturbed association with the DUB module and SAGA (Fig. 13). Ub-H2B levels in *sus1-10*-TAP and *sus1-12*-TAP backgrounds are slightly increased compared to wild-type, although their biochemical association with the DUB module appears intact. Correct Ubp8 function was previously shown to depend on the interaction with Sgf73, Sgf11 and Sus1 (Köhler et al., 2008). A slightly altered conformation of mutant Sus1 could allow for normal assembly into the DUB module but nevertheless have an influence on Ubp8 activity.

## 2.10 Cell growth is impaired in *sus1* mutant strains

Next, the functional consequences of Sus1 dissociation from its interacting partners were examined by analyzing the growth phenotypes of mutant *sus1* alleles. As described above, *sus1Δ* cells exhibit a mild growth defect at 30°C, while a temperature-sensitive phenotype develops when cells are grown at 37°C (Rodriguez-Navarro et al., 2004).



**Figure 19** Sus1 mutant alleles with defects in SAGA or TREX-2 association exhibit a temperature-sensitive growth defect

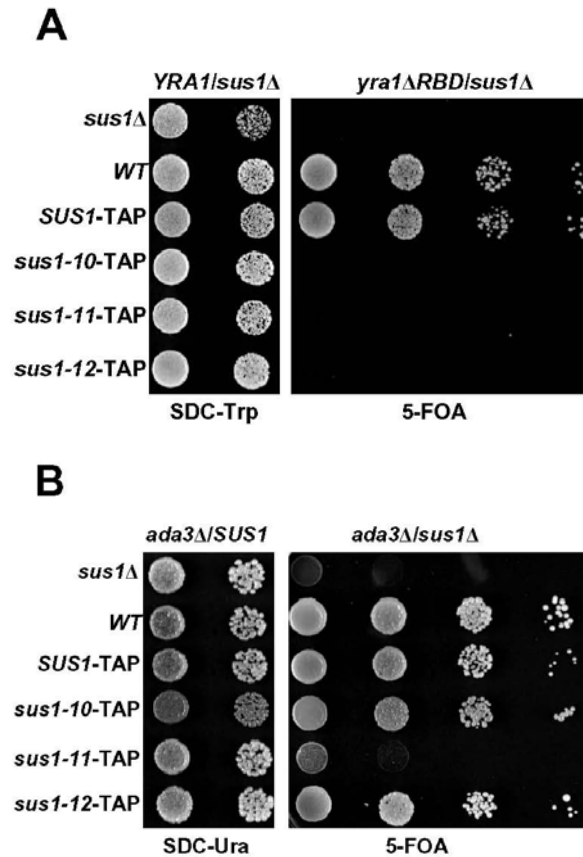
Growth analysis of cells carrying genomically integrated wild-type *SUS1*-TAP (*WT*-TAP) or the indicated mutants. The same number of cells was spotted to YPD plates in tenfold serial dilutions and incubated for 2 days at 30°C or 37°C. Wild-type and *sus1Δ* cells are shown for comparison.

While epitope-tagging of *SUS1* (*SUS1*-TAP) did not cause a growth defect compared to wild-type cells, all three mutants tested displayed significant retardation of growth at the restrictive temperature of 37°C (Fig. 19). Analogous to previous results, *sus1-11*-TAP showed the strongest effect, comparable to *sus1Δ* cells. The growth of *sus1-12*-TAP was slightly less affected than *sus1-10*-TAP, consistent with a greater extent of uncoupling from TREX-2 for the *sus1-10*-TAP allele.

In summary, the functional defects of the mutant alleles in SAGA or TREX-2 cause a significant growth defect at elevated temperatures.

## **2.11 Mutant *sus1* alleles differentially affect genetic interactions with factors involved in transcription or mRNA export**

The dual function of Sus1 as a member of the SAGA and of the TREX-2 complex is reflected in a widespread network of genetic interactions with key factors involved in transcription and mRNA export (Collins et al., 2007; Köhler et al., 2006; Köhler et al., 2008; Rodriguez-Navarro et al., 2004; S. Lutz and E. Hurt, unpublished data). To test how mutant *sus1* alleles would affect this genetic network, their growth phenotypes were analyzed in combination with a mutant allele of a factor involved in mRNA export (Yra1) or with a *bona fide* subunit of SAGA (Ada3). While interaction defects of mutant *sus1* alleles with SAGA were thought to negatively influence cell growth in combination with *ADA3*, the TREX-2 association defects that were shown to lead to impaired mRNA export could cause a growth defect in combination with the mutant allele of *YRA1*. *sus1Δ* was previously shown to be synthetically lethal in combination with the *yra1ΔRBD* allele (Rodriguez-Navarro et al., 2004) and synthetically enhanced in combination with *ada3Δ* (identified by S. Lutz, Hurt laboratory).



**Figure 20 Mutant *sus1* alleles differentially influence the genetic interactions of *SUS1***

Genomically integrated wild-type or mutant *sus1*-TAP in (A) a *yra1Δ* shuffle strain (carrying *YRA1* on a *URA3* plasmid) or (B) an *ada3Δ* shuffle strain (carrying *SUS1* on a *URA3* plasmid). *yra1Δ* strains were transformed with the *yra1ΔRBD* allele (*TRP1* plasmid). The same number of transformed cells was spotted to SDC-Trp or SDC-Ura plates (as loading control) and 5-FOA plates in tenfold serial dilutions and incubated for 2 days or 3 days (*ada3Δ* on 5-FOA) at 30°C. No growth on 5-FOA indicates synthetic lethality.

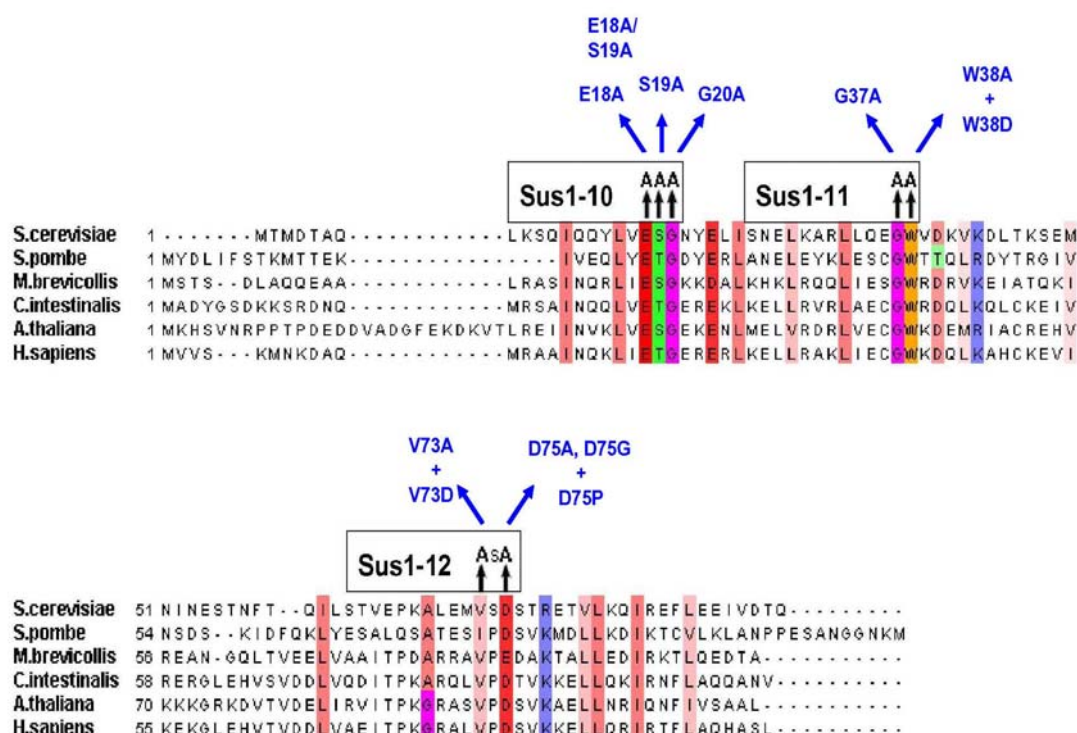
*sus1-11*-TAP shows severe growth retardation in combination with *yra1ΔRBD* as well as with *ada3Δ* which parallels the biochemical data indicating that this allele failed to interact with SAGA and TREX-2. *sus1-10*-TAP and *sus1-12*-TAP, on the other hand, do not show impaired growth in combination with *ada3Δ*. However, these alleles proved to be synthetically lethal with *yra1ΔRBD* (Fig. 20). This is consistent with their rather selective uncoupling from TREX-2 and observed functional defects of these alleles. Taken together, the genetic data underline the necessity of intact interaction of Sus1 with its ligands for an intact function of the protein. Moreover, the biochemical defects of the mutant *sus1* alleles are reflected by differential genetic defects that affect the mRNA export pathway alone (*YRA1*) or in combination with the transcription and chromatin remodelling pathway (*ADA3*).



## 2.12 Defects caused by the mutant alleles *sus1-11* and *sus1-12* can be attributed to single point mutations

In the preceding section, three mutants of Sus1 were characterized with respect to their function as part of the complexes SAGA and TREX-2. The results show that the mutations present in Sus1-11 lead to a loss of interaction with both TREX-2 and SAGA while for Sus1-10 and Sus1-12 the interaction with TREX-2 is selectively disturbed. All three mutant versions of Sus1 (Sus1-10, -11, -12) contain two or more mutated residues. Subsequently, we created single point mutations to narrow down the residues that are critical in Sus1 function.

In the course of generating these mutations, the crystal structure of Sus1 bound to Sac3<sup>CID</sup> was solved (for details of the Sac3<sup>CID</sup>:Cdc31:Sus1 structure, see Discussion and Jani et al., 2009). Four additional mutations of Sus1 were generated with respect to the novel structural data.



**Figure 21 Overview of the generated single point mutations of Sus1**

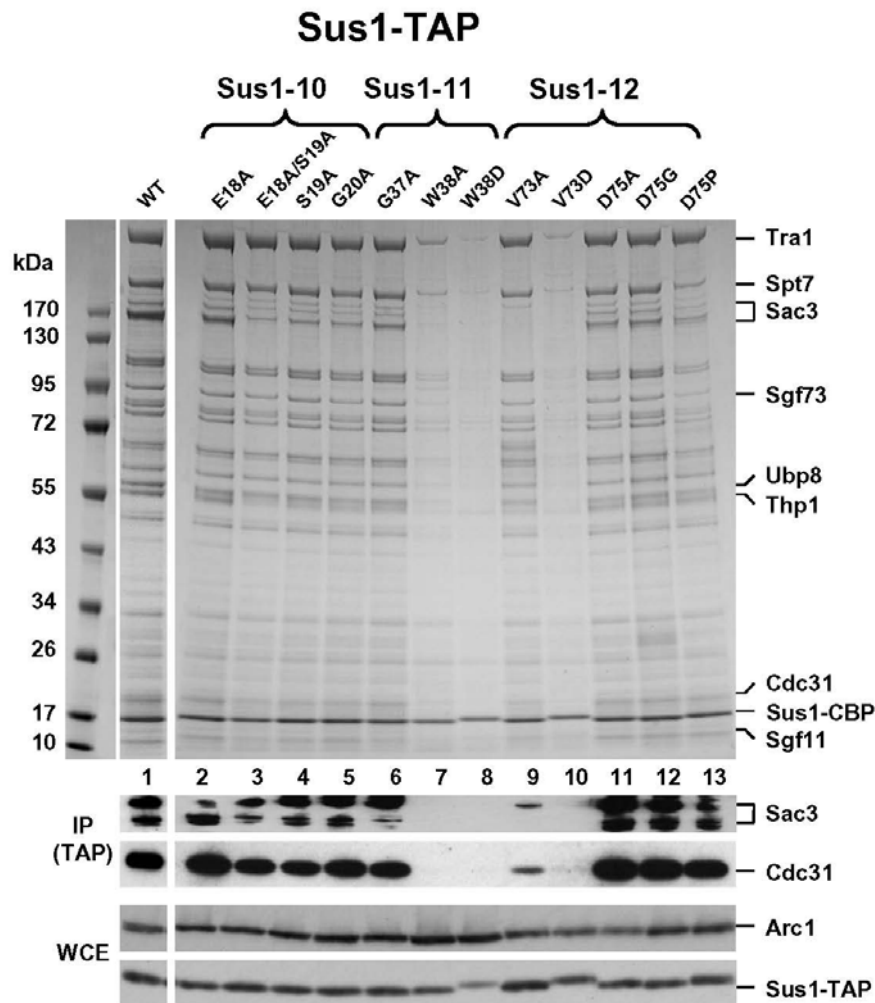
Protein sequence alignment is identical to the graph shown in Figure 9C. Black arrows indicate the individual conserved residues mutated to alanine in the mutants Sus1-10, Sus1-11 and Sus1-12 (shown in boxes). The newly generated separate mutations of conserved residues are indicated with blue arrows and depicted in blue.

The mutations of separate residues that were deduced from the combined alleles Sus1-10, Sus1-11 and Sus1-12 are E18A (glutamate 18 to alanine), E18A/S19A, G20A (derived from Sus1-10), G37A, W38A (derived from Sus1-11), V73A and D75A (derived from Sus1-12) as is shown in Fig. 21.

The additional mutations that were generated with respect to the solved crystal structure of the Sac3<sup>CID</sup>:Cdc31:Sus1 complex are W38D, V73D, D75G and D75P (Fig. 21). Aspartate at position 75 (D75) could be crucial for the correct formation of the turn between helix  $\alpha$ 4 and helix  $\alpha$ 5 (covered by the mutations of the allele Sus1-12). Due to the high rotational flexibility of aspartate, this residue is often found in turn regions of proteins (Hovmöller et al., 2002). To analyze the contribution of D75 to the formation of this turn, I generated mutations of this residue to proline (D75P) and to glycine (D75G).

As the Sac3<sup>CID</sup>-Sus1 interaction was shown to be mainly based on hydrophobic interactions (see Discussion), conserved hydrophobic residues (W38, V73) are likely to be involved in the binding of Sus1 to Sac3<sup>CID</sup>. These hydrophobic residues were replaced by the hydrophilic amino acid aspartate. Their replacement was thought to increase the effects seen with the mutations of these residues to alanine.

The newly generated Sus1 mutants were tested for interaction with SAGA and TREX-2.



**Figure 22 Single point mutations differentially contribute to the phenotypes of the combined mutants of *Sus1***

Affinity purification of genomically expressed *Sus1*-TAP from wild-type (WT) or indicated mutant *sus1* strains. Proteins were purified according to the TAP method (see Materials and Methods) and final eluates were analyzed by SDS-PAGE (4-12% gradient gel, MOPS buffer) and Coomassie staining. Bands are indicated according to the molecular mass of proteins reproducibly appearing in *Sus1*-TAP purifications and previously identified by mass spectrometry (Köhler et al., 2008). Purified protein eluates (TAP) were analyzed by Western blotting using  $\alpha$ -Sac3 and  $\alpha$ -Cdc31 antibodies. Expression levels of *Sus1*-TAP in whole cell extracts (WCE) were analyzed by Western blotting followed by protA detection (protein A is part of the TAP tag) and compared to Arc1 (yeast cytosolic marker protein). Original combined alleles of *sus1* comprising the individual mutations are shown above their derived mutants.

In contrast to the TAP results from *Sus1*-10 (Fig. 13, lane 2), the separate mutations E18A, E18A/S19A, S19A or G20A co-enriched subunits of both complexes, SAGA and TREX-2 (verified by Western analysis for Sac3 and Cdc31; Fig. 22, lanes 2-5). The affinity purification of the TAP-tagged mutants G37A and W38A, derived from *Sus1*-11, showed a co-purification pattern that differed from the combined mutant (Fig. 13, lane 3 and Fig. 22, lanes 6 and 7). While G37A-TAP co-purified TREX-2

and SAGA factors similar to wild-type Sus1-TAP, W38A-TAP caused a significant decrease in binding to TREX-2 and SAGA factors. However, W38A-TAP still co-purified residual amounts of SAGA subunits.

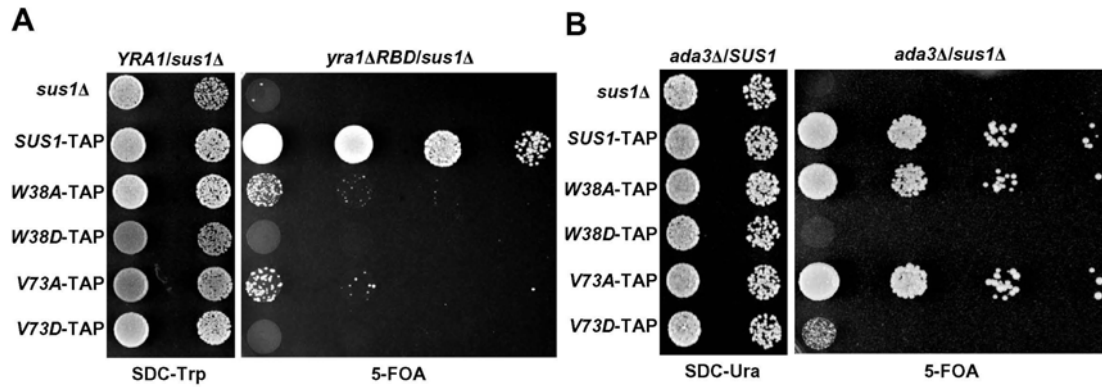
In the case of Sus1-12, the biochemical defects seem to be caused by only one of the two mutations present in the combined mutant. The mutation V73A leads to a reduced co-purification of TREX-2 subunits while the co-enrichment with typical SAGA factors remains largely unaffected (Fig. 22, lane 9). In contrast, D75A does not cause an apparent biochemical defect when affinity purified from yeast (Fig. 22, lane 11).

The mutation of the hydrophobic residues W38 or V73 to aspartate, thought to disrupt binding of Sus1 to TREX-2 (for V73D) or TREX-2 and SAGA (for W38D), both showed a loss of TREX-2 factors with a concomitant decrease in the amount of co-purified SAGA factors (Fig. 22, lanes 8 and 10), however not to the extent seen with Sus1-11.

The amino acid change of aspartate 75 to glycine (D75G) or proline (D75P) did not show association defects with SAGA or TREX-2 (Fig. 22, lane 12+13). While D75G-TAP produced a pattern of co-purified proteins comparable to the wild-type Sus1-TAP purification, D75P led to slightly decreased interaction with SAGA and TREX-2 subunits. Thus, selective defects of Sus1 binding to TREX-2, as seen in Sus1-12, cannot be generated by a mutation of aspartate 75 to glycine or proline.

In summary, the biochemical defects observed with the mutant alleles *sus1-10* and *sus1-11* can not be elicited by single or separate mutations but probably require a combination of these mutations. In contrast, the mutation V73A seems to be causing the uncoupling from TREX-2 observed with *sus1-12*. Further amino acid changes suggested by the crystal structure of the Sac3<sup>CID</sup>:Sus1 complex (D75G, D75P) did not show significant interaction defects with TREX-2 or SAGA.

The single residue mutations that produced a protein pattern significantly different from wild-type Sus1-TAP in affinity purifications (W38A-TAP, W38D-TAP, V73A-TAP, V73D-TAP; Fig. 22) were subsequently analyzed for their growth phenotypes in combination with *yra1 $\Delta$ ARB*D or *ada3 $\Delta$* , as described earlier.



**Figure 23 The influence of selected single point mutations on genetic interactions of *Sus1* matches their specific or general association defects with TREX-2 or SAGA**

Genomically integrated wild-type or mutant *SUS1*-TAP in (A) a *yra1Δ* shuffle strain (carrying *YRA1* on a *URA3* plasmid) or (B) an *ada3Δ* shuffle strain (carrying *SUS1* on a *URA3* plasmid). *yra1Δ* strains were transformed with the *yra1ΔRBD* allele (*TRP1* plasmid). The same number of cells was spotted to SDC-Trp or SDC-Ura plates (as loading control) and 5-FOA plates in tenfold serial dilutions and incubated for 2 days or 3 days (*ada3Δ* on 5-FOA) at 30°C. No growth on 5-FOA indicates synthetic lethality.

In combination with the *yra1ΔRBD* allele, the growth of *W38A*-TAP (derived from *sus1-11*) was impaired (Fig. 23A), while growth of *W38A*-TAP/*ada3Δ* was comparable to wild-type *SUS1*-TAP (Fig. 23B). Thus, the residual biochemical association of this mutant with SAGA seems to be sufficient to rescue growth in combination with *ada3Δ*. *W38D*-TAP, in contrast, led to synthetic lethality in both combinations tested, underlining the pronounced loss of association with SAGA and TREX-2 in this mutant. The allele *V73A*-TAP caused a pronounced growth defect in combination with *yra1ΔRBD* but did not affect growth in combination with *ada3Δ* and thus reflects the rather selective biochemical defects of this mutant with TREX-2. *V73D*-TAP finally impaired growth when combined with *yra1ΔRBD* but also in combination with *ada3Δ* which is consistent with the biochemical defects of this mutant.

Taken together, the analysis of the influence of single residue mutations of *SUS1* on genetic interactions in the context of transcription and mRNA export shows that *W38A*-TAP causes milder growth phenotypes than the allele *sus1-11*-TAP while the single residue mutation *V73A*-TAP indeed largely recapitulates the defects shown for *sus1-12*-TAP.

### 3. DISCUSSION

There is an emerging consensus that the processes of transcription and subsequent export of mRNA in yeast are tightly coupled to enable efficient gene expression (Brown and Silver, 2007; Köhler and Hurt, 2007; Luna et al., 2008; Luna et al., 2009; Perales and Bentley, 2009). An interesting example of a protein that functions in both processes is represented by Sus1, which acts as a functional subunit of the transcriptional co-activator SAGA as well as the nuclear pore-associated mRNA export complex TREX-2 (Rodriguez-Navarro et al., 2004). As part of SAGA, Sus1 is necessary for the formation and enzymatic activity of the histone H2B deubiquitinating module (Sgf73-Ubp8-Sgf11-Sus1) which is required for optimal transcription (Daniel et al., 2004; Köhler et al., 2008). Sus1 is also found to associate with the CID motif of Sac3, a crucial binding platform in TREX-2 which is responsible for the interaction of the complex with nuclear pores (Fischer et al., 2004).

Due to the association of Sus1 with SAGA and TREX-2, it was initially thought that Sus1 could bridge these two complexes and functionally couple transcription and mRNA export (Rodriguez-Navarro et al., 2004). Moreover, the repositioning of an activated gene locus to the nuclear periphery was shown to depend on Sus1 as well as other SAGA and TREX-2 subunits (Cabal et al., 2006). Understanding the characteristics of how Sus1 associates with SAGA or TREX-2 and how the protein functions in the respective complexes are important steps to clarify how transcription and mRNA export are integrated to provide optimal gene expression.

This study presents a comprehensive mutational analysis of Sus1 that successfully identified key residues for the interaction with TREX-2 or SAGA. Using novel Sus1 mutants that were selectively uncoupled from TREX-2, I was able to analyze the function of Sus1 in TREX-2 independent of its function in SAGA.

Results from this analysis help to understand how Sus1 could function at the interface of transcription and mRNA export.

### 3.1 Sus1 can be selectively uncoupled from TREX-2 by mutations in interhelical turn regions

Apart from extending the genetic network around the *SAC3<sup>CID</sup>* motif, this work successfully generated mutant alleles of Sus1 that lead to a specific disruption of the interaction with TREX-2 while the interaction with SAGA remained largely undisturbed or a general loss of binding to both complexes.

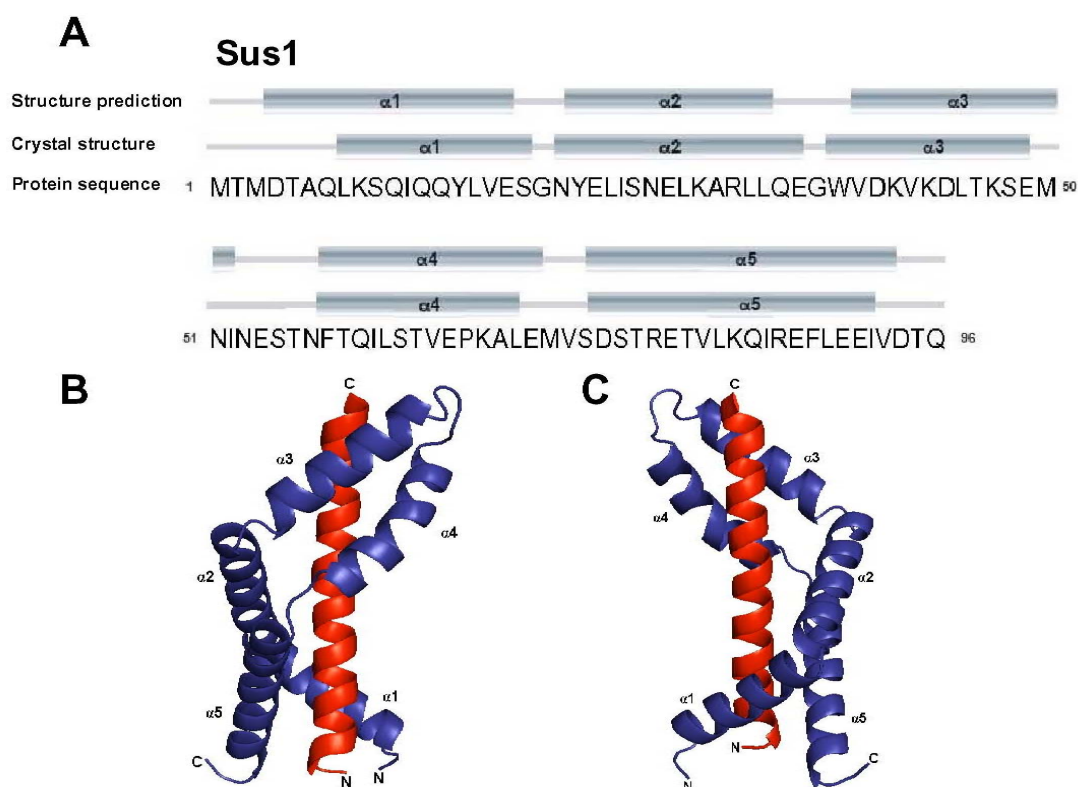
In the course of the mutagenesis of Sus1, it turned out that the less conserved N- or C-terminal parts of the protein (amino acids 1-10 or 91-96, Fig. 9A) can be deleted from the protein without significantly affecting the binding of Sus1 to SAGA and TREX-2. Furthermore, it could be shown that the systematic replacement of charged residues of Sus1 by alanine does not disturb the interaction with SAGA and TREX-2 which argues against a predominantly ionic interaction of Sus1 with its partner complexes.

Eventually, I was able to identify mutations that lead to an uncoupling of Sus1 from Sac3 (the binding partner in TREX-2) while leaving the interaction with Sgf11 (the binding partner in SAGA) largely intact. The mutations that cause this selective dissociation from TREX-2, via the *sus1-10* and *sus1-12* alleles, are clustered in putative turns between  $\alpha$ -helices predicted by sequence analysis. Interestingly, the alleles that were selectively uncoupled from TREX-2 contain mutations that are situated in the turns that connect the N-terminal helix (helix  $\alpha$ 1) or the C-terminal helix (helix  $\alpha$ 5) with the rest of the protein. In contrast, the *sus1-11* allele, in which two highly conserved residues of the central turn between helix  $\alpha$ 2 and helix  $\alpha$ 3 (glycine 37 and tryptophane 38) were mutated, was concomitantly impaired in its interaction with both TREX-2 and SAGA.

The selective or general defects observed with these mutants of Sus1 speak for a high relevance of the turn region residues for Sus1 function. The relevance of these residues for the interaction of Sus1 with TREX-2 or SAGA could either consist in direct molecular contacts to Sac3 or Sgf11 or a requirement of these particular residues for the correct conformation of Sus1 which is needed for binding to its partners. The exact contribution of Sus1 turn region residues to the association with TREX-2 can now be viewed in the context of the crystal structure of the Sac3<sup>CID</sup>-Cdc31-Sus1 complex that was recently solved.

### 3.2 The crystal structure of Sus1 bound to Sac3<sup>CID</sup> demonstrates how mutations of Sus1 affect this binding

In the course of this work, the crystal structure of Sus1 in a complex with Cdc31 and Sac3<sup>CID</sup> became available which revealed how Sus1 associates with Sac3. This novel data helps to put the mutations of Sus1 and their effects on Sus1 function in a structural context (Jani et al., 2009).



**Figure 24** Sus1 shows an articulated five-helix hairpin fold as was previously predicted by sequence analysis

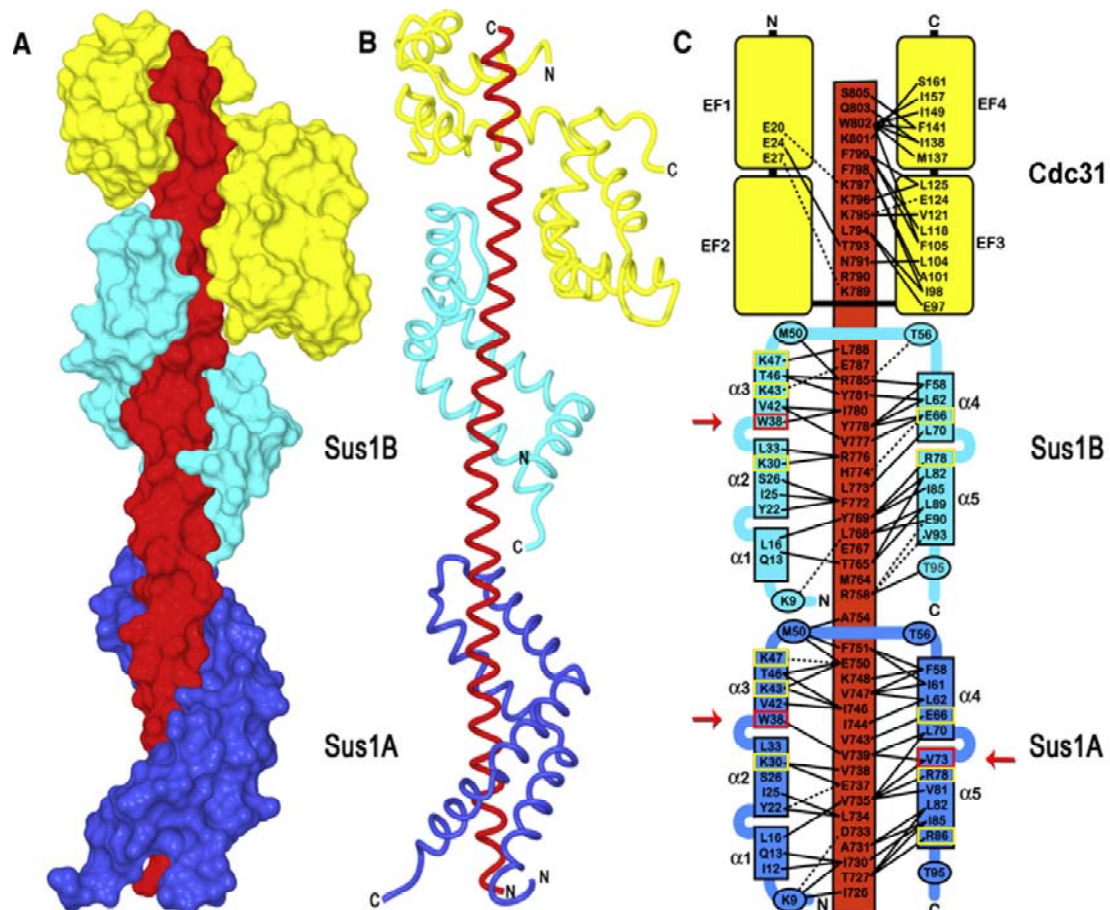
(A) Comparison of the predicted secondary structure of Sus1 with the crystal structure data. The predicted secondary structure of Sus1 (from Fig. 9B) and the secondary structure deduced from the crystal structure are aligned with and shown above the protein sequence of Sus1.  $\alpha$ -helices of Sus1 are numbered from the N-terminus to the C-terminus. (B) front and (C) rear view of one molecule of Sus1 (shown in blue) binding to the Sus1A binding site of Sac3 (aa 723-752; shown in red); N- and C-termini are indicated,  $\alpha$ -helices are labelled as in (A).

The secondary structure prediction that was originally done for Sus1 (Fig. 9B) was largely confirmed by the crystal structure (Fig. 24A). The fold of Sus1 was indeed based on five  $\alpha$ -helices that are separated by interhelical turns of varying size. The  $\alpha$ -helices of Sus1 arrange into an articulated antiparallel hairpin structure that curves



around the helix that is formed by the Sac3<sup>CID</sup> (Fig. 24B+C). This five-helix hairpin fold of Sus1 could represent a novel fold type as it does not appear to have close parallels in the protein structure database and is ideally suited to wrap around the Sac3<sup>CID</sup> helix. The interhelical turns (except for the turn between helix  $\alpha 3$  and  $\alpha 4$  which appears considerably disordered in the crystal structure) have an important role for the characteristic fold of Sus1, allowing the protein to curve around the helix formed by Sac3<sup>CID</sup>. This is corroborated by our previous observation that some residues that are situated in these interhelical turns are conserved among different species (Fig. 9C). The residue proline 67 of Sus1 introduces a kink in helix  $\alpha 4$  which further helps to wrap intimately around the Sac3 helix (Fig. 24B+C). The two arms of the antiparallel hairpin of Sus1 were mainly held together by hydrophobic interactions which involve, among others, the highly conserved residue tryptophane 38 (Jani et al., 2009).

The extended fold of the small protein Sus1 provides a surprisingly large surface area which is available for the interaction of Sus1 with its partner complexes SAGA or TREX-2. The structure of the whole Sac3<sup>CID</sup>:Cdc31:Sus1 complex reveals further details of the association of these proteins inside TREX-2.



**Figure 25** Crystal structure of the Sac3<sup>CID</sup>:Cdc31:Sus1 complex

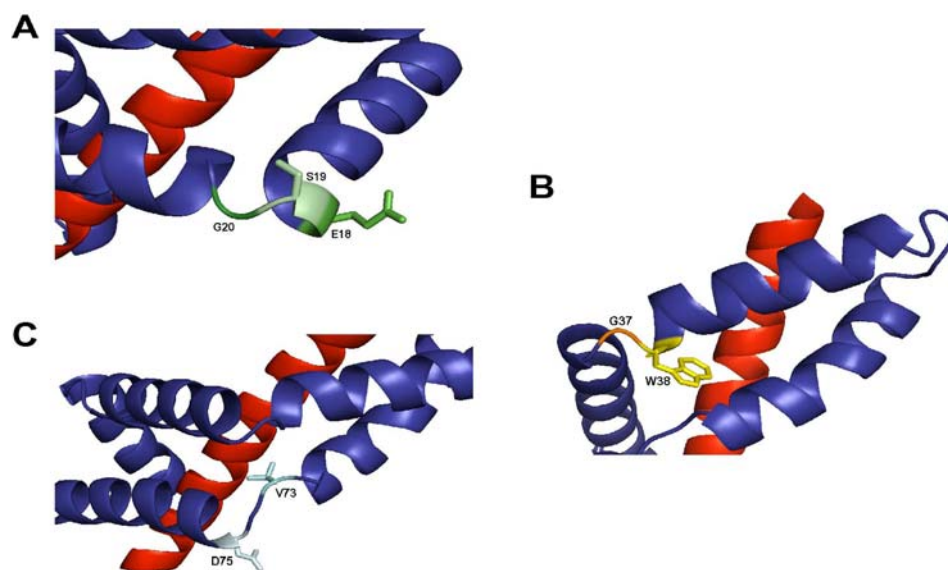
Overview of the crystal structure of the Sac3<sup>CID</sup>:Cdc31:Sus1 complex. (A) Surface representation of Cdc31 (yellow, on top) and two copies of Sus1 (Sus1B in cyan and Sus1A in blue; middle and bottom) bound to the Sac3<sup>CID</sup> helix (shown in red). (B) Secondary structure representation. (C) Residues involved in the interaction between Sac3 and its ligands. Principal interaction interfaces are depicted as solid lines, putative H bonds are depicted as dashed lines. Residues mutated in the course of the charge-to-alanine mutagenesis are highlighted by yellow boxes. The hydrophobic residues W38 and V73 are highlighted by red boxes and indicated by red arrows (adapted from Jani et al., 2009).

The Sac3<sup>CID</sup> forms an extended  $\alpha$ -helix with one Cdc31-binding site and two binding sites for Sus1 (Sus1A and Sus1B; Fig. 25). Interestingly, Sus1A and Sus1B as well as Sus1B and Cdc31 exhibit only negligible interaction with each other when bound to Sac3<sup>CID</sup> (Jani et al., 2009).

Cdc31 as well as the two molecules of Sus1 wrap around the elongated  $\alpha$ -helix that is formed by Sac3<sup>CID</sup> in a side-by-side fashion. In this complex, the binding of both copies of Sus1 to Sac3 was found to be mainly based on hydrophobic interactions (Fig. 25C). The large contact surface created by the wrapping of Sus1 around Sac3<sup>CID</sup> could explain the results from the charge-to-alanine mutagenesis. If residues along the whole sequence of Sus1 contribute to stable interaction with Sac3<sup>CID</sup>, the mutation of

a single charged residue or a cluster of charged residues of Sus1 involved in the interaction (highlighted in yellow in Fig. 25C) will not be sufficient to abolish the interaction.

A detailed inspection of the respective turn regions of Sus1 can potentially explain how the mutations of Sus1 could lead to a selective dissociation from TREX-2 or TREX-2 and SAGA.



**Figure 26** The crystal structure of Sus1 bound to Sac3<sup>CID</sup> reveals the involvement of key residues of Sus1 in the interaction with Sac3<sup>CID</sup>

Details from the structure of Sus1 (shown in dark blue) bound to the Sus1A binding site of Sac3<sup>CID</sup> (aa723-752; shown in red) with a focus on the (A) turn between helix  $\alpha 1$  and  $\alpha 2$  containing residues E18, S19 and G20 (shown in shades of green); (B) turn between helix  $\alpha 2$  and  $\alpha 3$  containing residues G37 (orange) and W38 (yellow); (C) turn between helix  $\alpha 4$  and  $\alpha 5$  containing residues V73 (light blue) and D75 (grey). Selected turn region residues are depicted with their respective side chains and indicated by the one-letter amino acid code and their position in the amino acid sequence of Sus1.

The residues in the turn region between helix  $\alpha 1$  and  $\alpha 2$  (E18, S19, G20) do not seem to be in close contact with the Sac3<sup>CID</sup>  $\alpha$ -helix (Fig. 26A). Therefore, it is unlikely that these residues are involved in the interaction with Sac3. Rather, the collective mutation of all three turn residues to alanine (as in Sus1-10) could considerably alter the conformation of the turn between helix  $\alpha 1$  and helix  $\alpha 2$  of Sus1. This might lead to a distorted angle of helix  $\alpha 1$  towards the rest of the structure, preventing the interaction of this helix with the Sac3<sup>CID</sup> and thus compromising the association of Sus1 with Sac3.

The side chain of the hydrophobic residue W38 in the turn region between helix  $\alpha 2$  and  $\alpha 3$  is oriented towards the Sac3<sup>CID</sup> helix and involved in intermolecular binding to both potential Sus1 binding sites of Sac3<sup>CID</sup> (Fig. 25C and 26B). Furthermore, this residue was suggested to have an additional intramolecular role and could be important to keep the hairpin fold of Sus1 together tightly (Jani et al., 2009). Indeed, a reduction or disruption of the hydrophobic character of this residue by a mutation to alanine or aspartate impaired binding to SAGA and TREX-2, presumably caused by a reduced affinity towards the binding sites in Sac3<sup>CID</sup> (Sus1A and Sus1B) and a concomitant weakening of the compact hairpin fold of Sus1. The involvement of W38 in intermolecular as well as intramolecular contacts serves to explain why the mutations W38A and W38D affect binding to SAGA and TREX-2 simultaneously. However, the combination of the mutations G37A and W38A seems to be needed to cause the striking loss of binding of Sus1 to both complexes, as in Sus1-11.

The hydrophobic residue V73, situated in the turn between helix  $\alpha 4$  and  $\alpha 5$ , was reported to be involved in binding to the Sus1A site but not to the Sus1B site (Fig. 25C; Jani et al., 2009). Functional data from the same study shows that a deletion of the Sus1A site does not lead to the severe defects observed when Sus1B is removed, suggesting a lesser involvement of the SUS1A binding site in robust Sac3<sup>CID</sup>-Sus1 interaction. The data presented in this study in contrast shows that the mutation of V73 to alanine leads to a significant decrease in association with Sac3<sup>CID</sup> and thus TREX-2 (see above). This indicates that the binding of Sus1 to the Sus1A binding site of Sac3 is indeed essential for the interaction with TREX-2.

The suspected role of the hydrophobic residue V73 for the interaction with Sac3<sup>CID</sup> could be corroborated by the available crystal structure of Sus1 bound to Sac3. Indeed, the side chain of V73 is oriented towards the Sac3<sup>CID</sup> helix (Fig. 26C) and was reported to interact with the elongated Sac3<sup>CID</sup> helix (Fig. 25C). The side chain of D75, the second residue mutated to alanine in Sus1-12, points away from the Sac3<sup>CID</sup> helix and is not involved in the interaction between the two proteins. A proposed requirement of this specific residue for correct turn formation at this position was contradicted by results that show that mutations of D75 to alanine, glycine or proline did not have a significant influence on binding to TREX-2 or SAGA.

Taken together, the results of this study indicate a differential relevance of the residues in turn regions of Sus1 for the binding to SAGA or TREX-2. The combined mutation Sus1-10 might cause a distorted orientation of helix  $\alpha 1$  which selectively

disturbs the interaction with TREX-2 but not SAGA. This would suggest that helix  $\alpha 1$  of Sus1 is more involved in the binding to Sac3<sup>CID</sup> than to Sgf11, as the interaction with Sgf11 and other SAGA factors remains largely undisturbed in this mutant. The hydrophobic residue V73 (in the turn between helix  $\alpha 4$  and  $\alpha 5$ ) was reported to make direct molecular contacts with Sac3<sup>CID</sup> and shown to be a key residue for the interaction with TREX-2 as its mutation to alanine (in Sus1-12 or V73A) produces selective defects of TREX-2 association. Although both Sus1-10 and V73A cause similar biochemical defects, the molecular basis of how these mutations selectively uncouple Sus1 from TREX-2 seem to be different. Lastly, the disturbed association of the combined mutant Sus1-11 with both partner complexes was shown to be mainly caused by a mutation of the residue W38, which is in direct hydrophobic contact with Sac3<sup>CID</sup> and also suggested to be important for the compact fold of Sus1 (Jani et al., 2009). This additional intramolecular role of W38 could explain the disturbed interaction with Sac3<sup>CID</sup> as well as with Sgf11 caused by a mutation of this residue. The fact that mutants selectively disturbing the binding of Sus1 to Sgf11 in the SAGA complex could not be identified might point to a different way of interaction of Sus1 with this complex through additional contacts with Ubp8 and Sgf73 in the DUB module. If residues in the sequence of Sus1 that are mainly involved in the binding to Sgf11 exist, solving the structure of Sus1 in complex with Sgf11 will help to identify those residues. Recently, the crystal structure of the Sus1:Sgf11 complex revealed the differences of the binding of Sus1 to Sgf11 compared to the binding to Sac3<sup>CID</sup> (Ellisdon et al., 2009).

### **3.3 The association of Sus1 with TREX-2 is crucial for targeting of the complex to NPCs and efficient mRNA export**

With the mutants Sus1-10 and Sus1-12 that selectively reduce binding to Sac3<sup>CID</sup> in TREX-2, powerful tools were made available to assay the function of Sus1 in this complex largely independent of its function in SAGA. During the analysis of these mutants of Sus1, their interference with TREX-2 functions was further corroborated. Sus1 was previously shown to be necessary to target the TREX-2 subunits Sac3 and Thp1 to the nuclear periphery. This targeting was suggested to depend on the function

of Sus1 in TREX-2 as the uncoupling of Sus1 from SAGA (in *ubp8Δ* cells) does not lead to mislocalization of Sac3-GFP or Thp1-GFP (Köhler et al., 2008).

However, the mutants described above permitted for the first time a direct analysis of the consequences of Sus1 dissociation from TREX-2 for the localization of the complex. This work demonstrates that alleles significantly impaired in interaction with TREX-2, while still being largely associated with SAGA, lead to a defective localization of Sac3-GFP to NPCs. In addition, a graded defect in TREX-2 association (more articulate for Sus1-10, less for Sus1-12) is reflected as a graded mislocalization of TREX-2 to the nucleoplasm and the cytoplasm. This confirms the essential role of Sus1 in TREX-2 for correct targeting to the nuclear periphery.

Another effect of a deletion of Sus1 that was predicted to be linked to its function in TREX-2 is a defect in exporting mRNA out of the nucleus (Rodriguez-Navarro et al., 2004). Correct mRNA export was previously shown to be closely linked to the localization of TREX-2 at nuclear pores (Fischer et al., 2002). Analogously, the disturbed association of mutant *sus1* alleles with TREX-2 was shown to cause a bulk mRNA export block. As in the biochemical analysis of assembly of the alleles into TREX-2 and nuclear pore localization of the complex, cells carrying *sus1-10* or *sus1-12* again show graded degrees of mRNA accumulation in the nucleus matching the different association defects of the alleles with TREX-2.

In summary, using the two mutants Sus1-10 and Sus1-12, it could be demonstrated that the loss of association of Sus1 with TREX-2 is reflected by the extent of mislocalization of TREX-2 from nuclear pores and a consequent mRNA export defect. These results further strengthen the previously suggested key role for the function of Sus1 in TREX-2 for NPC tethering of the complex and efficient mRNA export.

### **3.4 A model of Sus1 function between TREX-2 and SAGA**

Over the past years, multiple different studies have reported that a number of genes are dynamically repositioned to the nuclear periphery upon transcriptional activation (reviewed in Akhtar and Gasser, 2007; Köhler and Hurt, 2007) as suggested by the classic “gene gating” hypothesis (Blobel, 1985) which proposes that activated genes

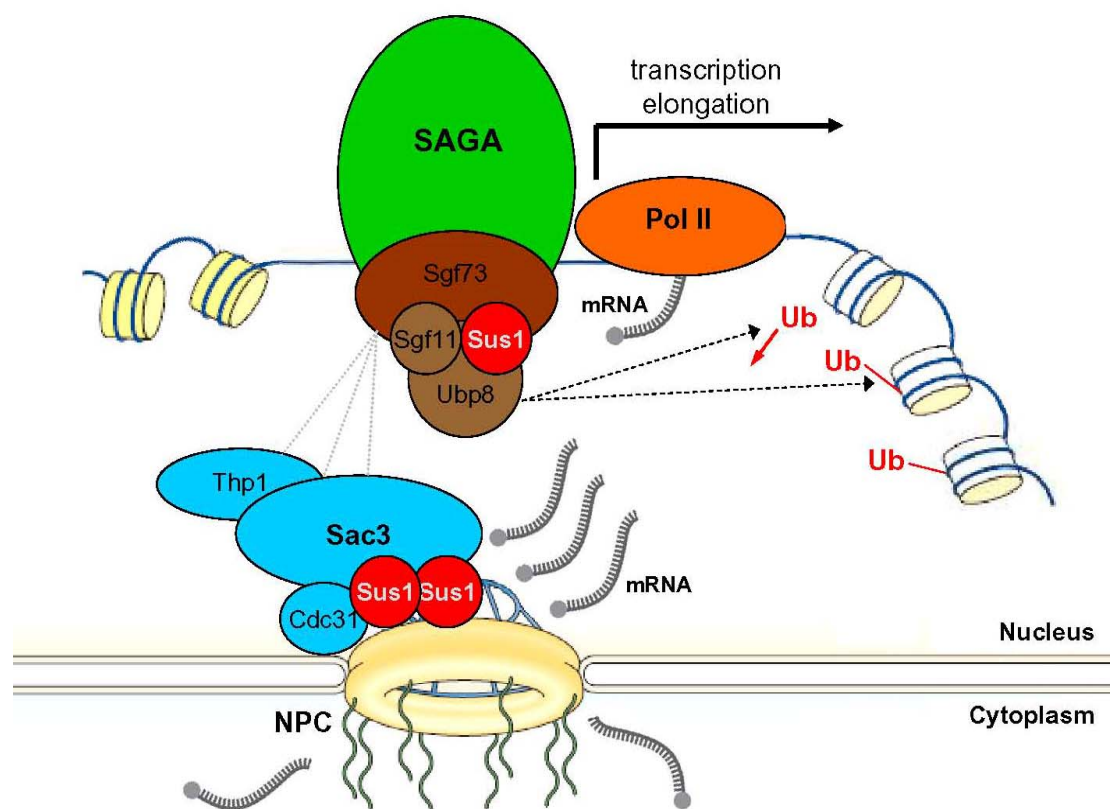
in the nucleus are gated (*i.e.* physically connected) to a particular NPC in the nuclear membrane. This connection appears to promote optimal transcription and efficient export of the nascent transcript from the nucleus (Brickner and Walter, 2004; Taddei et al., 2006). Although the molecular mechanisms of how activated genes can be targeted and subsequently tethered to the nuclear periphery are not fully understood, multiple factors have recently been reported to be involved in this repositioning. Among these factors is the nuclear basket protein Mlp1 which is also proposed to be involved in nuclear mRNA quality control (Galy et al., 2004; Vinciguerra et al., 2005). Mlp1 has been shown to mediate the association of active genes with the nuclear pore together with the general mRNA export receptor Mex67 (Dieppois et al., 2006) or subunits of the transcriptional co-activator SAGA (Luthra et al., 2007). Additional factors that were reported to be required for NPC localization of active genes include the nucleoporin Nup2 and the histone variant H2A.Z (Brickner et al., 2007), the Nup84 complex and the transcriptional regulator Rap1 (Menon et al., 2005) and the protein Sus1, which has a role in transcription as well as mRNA export.

Sus1 was initially found to be a member of both SAGA and TREX-2 and these complexes were reported to be physically connected (Rodriguez-Navarro et al., 2004). While Sus1 was recently shown to be important for dynamic repositioning of activated *GAL* genes and retention at the nuclear periphery (Cabal et al., 2006; Chekanova et al., 2008), the exact role of Sus1 in the functional coupling of transcription, gene gating and mRNA export is still unclear.

The central question that arises is if the roles of Sus1 in SAGA and TREX-2 are entirely separate from each other. Upon the identification of Sus1 as a subunit of SAGA as well as TREX-2, it was suggested that a SAGA-TREX-2 supercomplex might exist with Sus1 as the bridging factor (Rodriguez-Navarro et al., 2004). However, recent data from our lab indicate that SAGA-associated Sgf73 is necessary for a recruitment of Sac3 and Thp1 to SAGA and an interaction of SAGA and TREX-2 with each other (Köhler et al., 2008). Disruption of the DUB subcomplex in *ubp8Δ* cells on the other hand shows dissociation of Sus1 from SAGA but no significant effects on the composition of TREX-2. These data argue against a model of a SAGA-bound Sus1 being the sole bridging factor towards TREX-2.

Taking this into account, how could the dual role of Sus1 in SAGA and TREX-2 contribute to gene gating and transcription-coupled mRNA export? It was previously reported that Sus1 is required for the formation of the DUB module and its

deubiquitination activity *in vivo* and *in vitro* (Köhler et al., 2006; Köhler et al., 2008) and this activity was shown to be necessary for efficient transcriptional activation and elongation (Daniel et al., 2004; Henry et al., 2003; Wyce et al., 2007). This study presents similar findings for the role of Sus1 for TREX-2 complex function: While Sac3, Thp1 and Cdc31 can still interact with each other when Sus1 is uncoupled from TREX-2, the NPC targeting of the complex and efficient mRNA export is compromised in the absence of Sus1. These data suggest that both the DUB module as well as TREX-2 require the association with Sus1 for their respective function. Thus, Sus1 has an important role for the integration of optimal transcription and efficient mRNA export.



**Figure 27 The function of Sus1 in the SAGA and TREX-2 complexes mediates optimal transcription and efficient mRNA export**

As part of the DUB module (Sgf73-Ubp8-Sus1-Sgf11) within SAGA, Sus1 (shown in red) is required for the deubiquitination of histone H2B by Ubp8 (dashed arrows) which facilitates transcription elongation by RNA polymerase II and optimal transcription of messenger RNA. The assembly of Sus1 with the TREX-2 complex (Sac3-Thp1-Cdc31-Sus1) mediates targeting of this complex to the NPC and is required for efficient export of nascent mRNA. At the nuclear pore, SAGA-bound Sgf73 recruits the TREX-2 complex to SAGA (grey dotted lines) and might thus contribute to NPC repositioning of active genes. Through the formation of a SAGA-TREX-2-intermediate, the functions of Sus1 in transcription and mRNA export could be coordinated to provide optimal gene expression.



Upon transcriptional activation, SAGA is thought to form a transient intermediate with TREX-2 through the Sgf73-dependent recruitment of Sac3 and Thp1 to SAGA. This would bring the actively transcribed gene and the mRNA export machinery as well as the nuclear pore in close proximity with each other, allowing for the capture of nascent transcripts by the mRNA export complex TREX-2 and subsequent export out of the nucleus (Fig. 27). Because Sus1 is necessary for functional transcription elongation and mRNA export, a speculative model could involve a dynamic exchange of Sus1 between SAGA and TREX-2. Bound to SAGA, Sus1 would allow for optimal transcription of an activated gene and, upon transcription termination, the function of Sus1 in TREX-2 would ensure efficient export of nascent mRNAs out of the nucleus. The association of Sus1 with one or the other complex could be a way how different stages of gene expression are coordinated.

The recent determination of the crystal structure of a Sus1:Sgf11 subcomplex showing the differences and similarities of binding of Sus1 to Sgf11 or to Sac3<sup>CID</sup> will further elucidate the dynamics of Sus1 in transcription-coupled mRNA export (Ellisdon et al., 2009). In any case, the small protein Sus1, being able to associate with SAGA and TREX-2, appears to play an important role in transcription-coupled export and gene gating. The mutant alleles presented here constitute convenient and necessary tools to assay the individual contributions of Sus1 to transcription and mRNA export as well as their connection.

## 4. MATERIALS AND METHODS

### 4.1 DNA manipulation and plasmid cloning

Recombinant DNA techniques, like polymerase chain reaction (PCR), restriction digest of PCR fragments and plasmids, separation of DNA fragments by agarose gel electrophoresis and ligation of DNA fragments, as well as microbiological methods in *E. coli* like production of electrocompetent cells, electrotransformation with plasmids and propagation of cells, were done as previously described (Sambrook and Russell, 2001). All these DNA manipulation techniques were done in *Escherichia coli* DH5 $\alpha$  cells.

Plasmid DNA was isolated from *E. coli* using the Promega Wizard Plus SV Minipreps DNA purification system and DNA fragments from PCR amplifications and agarose gels were purified using the Wizard SV Gel and PCR clean-up system (Promega, Madison, USA).

Restriction enzymes were obtained from Fermentas (St. Leon-Rot, Germany). The system employed for PCR amplifications was Phusion High Fidelity DNA polymerase (Finnzymes, Espoo, Finland). Oligonucleotides were synthesized by biomers (Ulm, Germany) and Eurofins MWG Operon (Ebersberg, Germany) and DNA sequencing was also provided by Eurofins MWG Operon (Ebersberg, Germany).

#### 4.1.1 Introduction of internal point mutations by a two-step PCR method

For the directed mutagenesis of nucleotides of *SUS1* that are positioned inside the gene, a two-step PCR method adapted from a previously described protocol (Horton et al., 1989) was employed.

In the first PCR, two primary products are generated that comprise the 5' end or the 3' end of the gene with the introduced mutation. Both also include overlapping sequences to be able to anneal with each other at their 5' or 3' termini, respectively. After separation of the primary products from the original template DNA, two external primers (SUS1-TAP-for and SUS1-TAP-rev) extend the primary products

and amplify the complete DNA length including the internally mutagenized residues in a second PCR. The resulting DNA fragment containing 499 base pairs of the 5'UTR, the *SUS1* gene with a TAP tag and 350 base pairs of the 3'UTR could subsequently be cloned using *XhoI* and *NotI* sites.

#### 4.1.2 Plasmids used in this study

Plasmids used in this study are listed in table I. The following plasmids were generated during this study as follows: *SPE3* was amplified from genomic DNA of a yeast wild-type strain (W303) with ~500 base pairs of its 5'UTR and 3'UTR and cloned into the *PstI* and *XhoI* sites of pRS315 (pRS315-VPS53 was created analogously with *HindIII* and *ApaI* sites). The mutations D168A and Q181\* of *SPE3* were generated according to 5.1.2 and cloned into *PstI* and *XhoI* sites. Truncations of *SUS1* in pNOPPATA1L were generated by amplifying full-length or truncated versions of *SUS1* (N1-3, C1-3) from pRS424-SUS1-cDNA and inserting the DNA fragments in *BamHI*/*NcoI* sites of pNOPPATA1L. The plasmid pFA6a-klURA3 was generated by inserting klURA3 (amplified with URA3-for and URA3-rev oligos) from pBS1539 into *BglIII*/*EcoRI* sites of pFA6a-KANMX6. For a plasmid containing a TAP-tagged version of *SUS1* with the natural promoter and terminator, *SUS1* was amplified with 499 base pairs of its 5'UTR from a genomic DNA template of a *S. cerevisiae* Sus1-TAP strain and cloned into *XhoI*/*NotI* sites of pRS315. Following this, 350 base pairs of *SUS1* 3'UTR were amplified and cloned into *PstI* and *BamHI* (upstream of *NotI*) sites. The resulting P<sub>SUS1</sub>-SUS1-TAP-T<sub>SUS1</sub> was easily excisable using *XhoI* and *NotI* restriction enzymes. FLAG-tagged versions of *SUS1* were generated by a PCR using an oligo replacing the TAP-tag by a FLAG-tag and a novel *PstI* site. This DNA fragment was cloned into *XhoI* and *PstI* sites of pRS315-SUS1-TAP resulting in a P<sub>SUS1</sub>-SUS1-FLAG-T<sub>SUS1</sub>.

Site-directed mutagenized versions of *SUS1*-TAP and *SUS1*-FLAG were generated as described in 5.1.2 and subsequently cloned into *XhoI* and *NotI* sites of pRS315.

Plasmids for heterologous expression in *E. coli* were generated by amplifying wild type or mutant *sus1* from cDNA of *SUS1* and fusing the product in frame behind a hexa-histidine tag into *NcoI* and *BamHI* sites of a pET9d-6His vector.

The correctness of all plasmids generated was verified by sequencing.

Table I: **Plasmids used in this study**

Name	Relevant information	Reference
pRS315	<i>ARS/CEN LEU2</i>	Sikorski and Hieter, 1989
pHT4467-SAC3	<i>ARS/CEN6 (instable) URA3 ADE3 SAC3</i>	lab collection
pRS315-SAC3	<i>ARS/CEN LEU2 SAC3</i>	Fischer et al., 2002
pRS315-sac3ΔCID	<i>ARS/CEN LEU2 sac3Δaa733-851</i>	Fischer et al., 2004
pRS315-VPS53	<i>ARS/CEN LEU2 P<sub>VPS53</sub> VPS53 T<sub>VPS53</sub></i>	This study
pRS315-BUR6	<i>ARS/CEN LEU2 BUR6</i>	lab collection
pRS315-SPE3	<i>ARS/CEN LEU2 P<sub>SPE3</sub> SPE3 T<sub>SPE3</sub></i>	This study
pRS315-spe3D168A	<i>ARS/CEN LEU2 P<sub>SPE3</sub> spe3 (D168A) T<sub>SPE3</sub></i>	This study
pRS424-SUS1 (cDNA)	<i>ARS/CEN LEU2, SUS1 (cDNA)</i>	lab collection
pNOPTAP-SUS1 (FL)	<i>ARS/CEN LEU2, P<sub>NOPI</sub> N-terminal TAP tag, SUS1 (cDNA) aa 1-96, T<sub>ADHI</sub></i>	This study
pNOPTAP-SUS1 (N1)	<i>ARS/CEN LEU2 P<sub>NOPI</sub> N-terminal TAP tag, SUS1 (cDNA) aa 11-96, T<sub>ADHI</sub></i>	This study
pNOPTAP-SUS1 (N2)	<i>ARS/CEN LEU2 P<sub>NOPI</sub> N-terminal TAP tag, SUS1 (cDNA) aa 36-96, T<sub>ADHI</sub></i>	This study
pNOPTAP-SUS1 (N3)	<i>ARS/CEN LEU2 P<sub>NOPI</sub> N-terminal TAP tag, SUS1 (cDNA) aa 58-96, T<sub>ADHI</sub></i>	This study
pNOPTAP-SUS1 (C1)	<i>ARS/CEN LEU2 P<sub>NOPI</sub> N-terminal TAP tag, SUS1 (cDNA) aa 1-90, T<sub>ADHI</sub></i>	This study
pNOPTAP-SUS1 (C2)	<i>ARS/CEN LEU2 P<sub>NOPI</sub> N-terminal TAP tag, SUS1 (cDNA) aa 1-72, T<sub>ADHI</sub></i>	This study
pNOPTAP-SUS1 (C3)	<i>ARS/CEN LEU2 P<sub>NOPI</sub> N-terminal TAP tag, SUS1 (cDNA) aa 1-57, T<sub>ADHI</sub></i>	This study
pRS315-SUS1-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> SUS1-TAP T<sub>SUS1</sub></i>	This study
pRS315-sus1-10-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1 (E18A, S19A, G20A)-TAP T<sub>SUS1</sub></i>	This study
pRS315-sus1-11-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1 (G37A, W38A)-TAP T<sub>SUS1</sub></i>	This study
pRS315-sus1-12-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1 (V73A, D75A)-TAP T<sub>SUS1</sub></i>	This study
pRS315-sus1-E18A-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1 (E18A)-TAP T<sub>SUS1</sub></i>	This study
pRS315-sus1-E18A/S19A-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1 (E18A, S19A)-TAP T<sub>SUS1</sub></i>	This study
pRS315-sus1-G20A-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1 (G20A)-TAP T<sub>SUS1</sub></i>	This study
pRS315-sus1-G37A-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1 (G37A)-TAP T<sub>SUS1</sub></i>	This study

pRS315-sus1-W38A-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1</i> (W38A)-TAP T <sub>SUS1</sub>	This study
pRS315-sus1-W38D-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1</i> (W38D)-TAP T <sub>SUS1</sub>	This study
pRS315-sus1-V73A-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1</i> (V73A)-TAP T <sub>SUS1</sub>	This study
pRS315-sus1-V73D-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1</i> (V73D)-TAP T <sub>SUS1</sub>	This study
pRS315-sus1-D75A-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1</i> (D75A)-TAP T <sub>SUS1</sub>	This study
pRS315-sus1-D75G-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1</i> (D75G)-TAP T <sub>SUS1</sub>	This study
pRS315-sus1-D75P-TAP	<i>ARS/CEN LEU2 P<sub>SUS1</sub> sus1</i> (D75P)-TAP T <sub>SUS1</sub>	This study
pRS316-SUS1	<i>ARS/CEN URA3 P<sub>SUS1</sub> SUS1</i> T <sub>SUS1</sub>	This study
pRS316-SUS1-TAP <sup>##</sup>	<i>ARS/CEN URA3 P<sub>SUS1</sub> SUS1</i> -TAP T <sub>SUS1</sub>	This study
pRS316-SUS1-FLAG	<i>ARS/CEN URA3 P<sub>SUS1</sub> SUS1</i> -FLAG T <sub>SUS1</sub>	This study
pRS316-sus1-10-FLAG	<i>ARS/CEN URA3 P<sub>SUS1</sub> sus1</i> (E18A, S19A, G20A)-FLAG T <sub>SUS1</sub>	This study
pRS316-sus1-11-FLAG	<i>ARS/CEN URA3 P<sub>SUS1</sub> sus1</i> (G37A, W38A)-FLAG T <sub>SUS1</sub>	This study
pRS316-sus1-12-FLAG	<i>ARS/CEN URA3 P<sub>SUS1</sub> sus1</i> (V73A, D75A)-FLAG T <sub>SUS1</sub>	This study
pRS316-YRA1	<i>ARS/CEN YRA1</i>	Strässer and Hurt, 2000
pRS314- <i>yra1</i> ΔRRM	<i>ARS/CEN TRP1 yra1</i> ΔRRM	Strässer and Hurt, 2001
pFA6a-TAP-natNT2	natNT2, TAP tag, T <sub>ADHI</sub> ; for genomic C-terminal tagging	Kressler et al., 2008
pFA6a-klURA3	URA3 from <i>K. lactis</i> ; for deletion disruption	This study
pBS1539	URA3 from <i>K. lactis</i> , TAP tag	Rigaut et al., 1999
pGEX-TEV-SAC3 (573-805)	Amp <sup>r</sup> GST-TEV-sac3 (aa573-805)	generated by A. Köhler, Hurt lab
pET30a-TEV-CDC31	Kan <sup>r</sup> CDC31	lab of M. Stewart, Cambridge
pET24d-GST-TEV-SGF11	Kan <sup>r</sup> GST-TEV-SGF11	generated by A. Köhler, Hurt lab
pET9d-6His-SUS1	Kan <sup>r</sup> SUS1 (from cDNA)	This study
pET9d-6His-sus1-10	Kan <sup>r</sup> SUS1 (from cDNA) (E18A, S19A, G20A)	This study
pET9d-6His-sus1-11	Kan <sup>r</sup> SUS1 (from cDNA) (G37A, W38A)	This study
pET9d-6His-sus1-12	Kan <sup>r</sup> SUS1 (from cDNA) (V73A, D75A)	This study

### 4.1.3 Oligonucleotides used in this study

All oligonucleotides shown in Table II were synthesized by biomers, Ulm, Germany or Eurofins MWG Operon, Ebersberg, Germany. Primers used to synthesize the coding strand were named 5', primers to synthesize the noncoding strand were named 3'. Directed point mutations that were introduced using the respective nucleotides that were used to introduce directed point mutations in the PCR product are written in lower case letters, all others in upper case letters.

Table II: Oligonucleotides used in this study

Name	Sequence	Direction
SPE3-for	GTTCTTCTGCAGACACAAACGTTTCCTTAATAG	5'
SPE3-rev	GAAGTCGAGATTTATCAAATAATCGATATATGATTTC	3'
VPS53-for	GTAAATAAGCTTGTATTTGTATTTGTATGTG	5'
VPS53-rev	CTTCAAAGGGGCCCCAATAGCACATTTAATG	3'
spe3D168A-for	CATTTGACGTAATCATTACTGCCTCTTCTGACCCAGAAG	5'
spe3D168A-rev	CTTCTGGGTCAGAAGAGGCAGTAATGATTACGTCAAATG	3'
URA3-for	CATTGCAGATCTGGAGACAATCATATGGGAGAAGC	5'
URA3-rev	CATTGCGAATTCGAGCTCGCTTGTGTTCCTTACCATTAA GTTG	3'
SUS1-TAP-for	AGTCTCGAGCGTCTACACCACCGGAATTCTAG	5'
SUS1-TAP-rev	GTTCCGCGGCCGCTCTAGAACTAG	3'
SUS1 (cDNA)- for	GACTTGCCATGGAGATGACTATGGATACTGCGC	5'
SUS1 (cDNA)- rev	GTCGACGGATCCTCATTGTGTATCTACAATC	3'
sus1-C1-rev	GTCGACGGATCCTCATTCAAGAAATTCCTTATTTGC	3'
sus1-C2-rev	GTCGACGGATCCTCACATTTCTAATGCTTTGGGTTC	3'
sus1-C3-rev	GTCGACGGATCCTCAGTTCGTAGATTCATTTATG	3'
sus1-N1-for	GACTTGCCATGGAGATGCAACAATATCTTGTAG	5'
sus1-N2-for	GACTTGCCATGGAGATGGAAGGTTGGGTGGATAAAG	5'
sus1-N3-for	GACTTGCCATGGAGATGTTTACCCAAATTTATCC	5'
sus1-1-for	AGAATTTCAAACGcACTAgcAGCCAGACTACTTCAAGAA G	5'
sus1-1-rev	CTTCTTGAAGTAGTCTGGCTgcTAGTgCGTTTGAAATTCT	3'
sus1-2-for	CGAACTAAAAGCCgcACTACTTCAAGcAGGTTGGGTGGA T	5'

sus1-2-rev	ATCCACCCAACCTgCTTGAAGTAGTgcGGCTTTTAGTTCG	3'
sus1-3-for	CTTCAAGAAGGTTGGGTGGcTgcAGTCAAAGATTTGACC	5'
sus1-3-rev	GGTCAAATCTTTGACTgcAgCCACCCAACCTTCTTGAAG	3'
sus1-4-for	GGTTGGGTGGATAAAGTCgcAGcTTTGACCAAATCAGAG	5'
sus1-4-rev	CTCTGATTTGGTCAAAgCTgcGACTTTATCCACCCAACC	3'
sus1-5-for	GTCAAAGATTTGACCgcATCAGcGATGAACATAAATG	5'
sus1-5-rev	CATTTATGTTTCATCgCTGATgcGGTCAAATCTTTGAC	3'
sus1-6-for	CAAATTTTATCCACCGTAGcACCCgcAGCATTAGAAATGG	5'
sus1-6-rev	CCATTTCTAATGCTgcGGGTgCTACGGTGGATAAAATTTG	3'
sus1-7-for	GAATTTTTTTTTCTAGcAATGGTATCGGcTTCAACAAGG	5'
sus1-7-rev	CCTTGTTGAAGcCCGATACCATTgCTAGAAAAAAAAAATTC	3'
sus1-8-for	GGTATCGGATTCAACAgcGGcAACCGTTTTAAAGC	5'
sus1-8-rev	GCTTTAAAACGGTtgCCgcTGTGAATCCGATACC	3'
sus1-9-for	GGAAACCGTTTTAgcGCAAATAgcGGcATTTCTTGAAGAG	5'
sus1-9-rev	CTCTTCAAGAAATgCCgcTATTTGCgcTAAAACGGTTTCC	3'
sus1-10-for	CAACAATATCTTGTAGcAgCAGcAAACTATGAACTGTATG	5'
sus1-10-rev	CATACAGTTCATAGTTTgCTGcTgCTACAAGATATTGTTG	3'
sus1-11-for	CCAGACTACTTCAAGAAGcTgcGGTGGATAAAGTCAAAG	5'
sus1-11-rev	CTTTGACTTTATCCACCgcAgCTTCTTGAAGTAGTCTGG	3'
sus1-12-for	GAATTTTTTTTTCTAGAAATGGcATCGGcTTCAACAAGG	5'
sus1-12-rev	CCTTGTTGAAGcCCGATgCCATTTCTAGAAAAAAAAAATTC	3'
sus1(E18A)-for	CAACAATATCTTGTAGcATCAGGAACTATGAACTGTAT G	5'
sus1 (E18A)-rev	CATACAGTTCATAGTTTCCTGATgCTACAAGATATTGTTG	3'
sus1 (E18A/S19A)- for	CAACAATATCTTGTAGcAgCAGGAACTATGAACTGTAT G	5'
sus1 (E18A/S19A)- rev	CATACAGTTCATAGTTTCCTGcTgCTACAAGATATTGTTG	3'
sus1 (S19A)-for	CAACAATATCTTGTAGAAgCAGGAACTATGAACTGTAT G	5'
sus1 (S19A)-rev	CATACAGTTCATAGTTTCCTGcTTCTACAAGATATTGTTG	3'
sus1 (G20A)-for	CAACAATATCTTGTAGAATCAGcAAACTATGAACTGTAT G	5'
sus1 (G20A)-rev	CATACAGTTCATAGTTTgCTGATTCTACAAGATATTGTTG	3'
sus1 (G37A)-for	CCAGACTACTTCAAGAAGcTTGGGTGGATAAAGTCAAAG	5'
sus1 (G37A)-rev	CTTTGACTTTATCCACCCAAGcCTTCTTGAAGTAGTCTGG	3'
sus1 (W38A)-for	CCAGACTACTTCAAGAAGGTgcGGTGGATAAAGTCAAAG	5'

sus1 (W38A)-rev	CTTTGACTTTATCCACCgcACCTTCTTGAAGTAGTCTGG	3'
sus1 (W38D)-for	CCAGACTACTTCAAGAAGGTgacGTGGATAAAGTCAAAG	5'
sus1 (W38D)-rev	CTTTGACTTTATCCACgtcACCTTCTTGAAGTAGTCTGG	3'
sus1 (V73A)-for	GAATTTTTTTTTTCTAGAAATGGcATCGGATTCAACAAGG	5'
sus1 (V73A)-rev	CCTTGTTGAATCCGATgCCATTTCTAGAAAAAAAAAATTC	3'
sus1 (V73D)-for	GAATTTTTTTTTTCTAGAAATGGatTCGGATTCAACAAGG	5'
sus1 (V73D)-rev	CCTTGTTGAATCCGAatCCATTTCTAGAAAAAAAAAATTC	3'
sus1 (D75A)-for	GAATTTTTTTTTTCTAGAAATGGTATCGGcTTCAACAAGG	5'
sus1 (D75A)-rev	CCTTGTTGAAGcCGATACCATTTCTAGAAAAAAAAAATTC	3'
sus1 (D75G)-for	GAATTTTTTTTTTCTAGAAATGGTATCGGgTTCAACAAGG	5'
sus1 (D75G)-rev	CCTTGTTGAAcCCGATACCATTTCTAGAAAAAAAAAATTC	3'
sus1 (D75P)-for	GAATTTTTTTTTTCTAGAAATGGTATCGccTTCAACAAGG	5'
sus1 (D75P)-rev	CCTTGTTGAAggCGATACCATTTCTAGAAAAAAAAAATTC	3'
6xHIS-SUS1-for	CTAGCCCATGGGGATGACTATGGATACTGCGCAATTAAA G	5'
6xHIS-SUS1-rev	CAGAGTGGATCCTCATTGTGTATCTACAATCTCTTC	3'

## 4.2 Genetic methods

### 4.2.1 Growth media for *S.cerevisiae* and *E.coli*

*E.coli* cells were grown at 37°C and 150 rpm in liquid culture of LB medium (0.5% yeast extract, 1% tryptone, 0.5% sodium chloride) supplemented with antibiotics to select for respective plasmids (100 µg/ml ampicillin, 30 µg/ml kanamycin, 34 µg/ml chloramphenicol) or on agar plates made accordingly. *E. coli* cells used for expression of heterologous proteins were grown in minimal medium (0.2% glucose, 0.4% casamino acids).

Liquid cultures of *S. cerevisiae* cells were grown at 30°C (if not indicated otherwise) and 120 rpm in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose) or selective synthetic medium (2% glucose, 0.67% yeast nitrogen base) supplemented with all missing amino acids except the ones to select for. The final concentration of amino acids was set as previously described (Sherman, 1991). For a selection against



the *URA3* gene, SDC+all was additionally supplemented with 2 mg/ml of 5-fluoroorotic acid (5-FOA). The *URA3* gene product can convert 5-FOA into the toxic substance 5-fluorouracil, causing cell death.

Antibiotics used for selection in yeast cells were geneticin (0.2 mg/ml) and nourseothricin (clonNAT; 0.1 µg/ml). For induction of sporulation I incubated the cells on YPA (1% yeast extract, 2% peptone, 1% potassium acetate) or SPOR (0.022 % raffinose, 0.3% potassium acetate) plates. Tetrad dissection was performed using a Singer MSM micromanipulator.

Cell density in liquid culture was determined by measuring light absorption at 600 nm (OD<sub>600</sub>).

#### 4.2.2 Genomic gene replacement

To replace genomic wild-type *SUS1* with untagged and TAP-or FLAG-tagged alleles of *SUS1*, we used a strategy based on the fact that it is possible to select for (on -Ura plates) and also against the *URA3* gene (on 5-FOA plates).

First, we sought to obtain haploid W303 (MATa and MATα) *sus1::klURA3* (*URA3* gene from *K. lactis*) deletion disruption strain by sporulation and tetrad dissection of a heterozygous diploid *SUS1/sus1::klURA3*. The original heterozygous diploid strain was created by transformation of the diploid wild-type W303 strain with a *klURA3* PCR cassette (from pFA6a-*URA3*) that was flanked by short sequences homologous to *SUS1* 5' and 3'UTR, respectively.

The haploid *sus1::URA3* (MATa/α) strains were then co-transformed with integration cassettes of TAP- or FLAG-tagged wild-type and mutant versions of *SUS1* (cassettes were excised by *XhoI/BamHI* digestion of pRS315-*SUS1/sus1*-TAP or pRS316-*SUS1/sus1*-FLAG) and 100 ng of empty pRS315 for selection of transformants on -Leu plates. Living clones on SDC-Leu were subsequently replica-plated to 5-FOA containing plates to select for clones that had lost the *klURA3* gene due to site-specific recombination of the integration cassette with the original *SUS1* gene locus. To verify the correct integration of the various versions of *SUS1* or *sus1* and complete genomic loss of *klURA3*, yeast clones were analyzed by colony PCR and sequencing.

### 4.2.3 Yeast strains and basic yeast methods

Yeast strains used in this study are shown in Table III. Transformation of yeast cells with plasmid DNA was done as previously described (Ito et al., 1983). Plasmid recovery and genomic DNA preparation from yeast cells was performed as previously described (Hoffman and Winston, 1987).

Genomic deletion disruption and C-terminal TAP-tagging of genes was carried out as previously described (Janke et al., 2004; Kressler et al., 2008; Longtine et al., 1998).

Table III: Yeast strains used in this study

Name	Genotype	Reference
sac3 $\Delta$ sl	<i>MATa his3-11, 15, ura3-52, leu2-3, 112, trp1-1, ade2-1, ade3, sac3::kanMX4</i> (pHT4467- <i>SAC3</i> )	generated by A. Racz and T. Fischer, Hurt lab
sac3 $\Delta$ spe3 $\Delta$	<i>MATa his3-11, 15, ura3-52, leu2-3, 112, trp1-1, ade2-1, ade3, sac3::kanMX4, spe3::natNT2</i> (pHT4467- <i>SAC3</i> )	This study
SAC3-GFP	<i>MATa ade2-1, ade3, his3-11, 15, ura3-52, leu2-3, 112, trp1-1, SAC3-GFP::TRP1</i>	Cabal et al., 2006
SAC3-GFP <i>sus1</i> $\Delta$	<i>sus1</i> $\Delta$ ::klURA3 in <i>SAC3</i> -GFP	This study
SAC3-GFP <i>SUS1</i> -TAP	<i>SUS1</i> -TAP in <i>SAC3</i> -GFP	This study
SAC3-GFP <i>sus1</i> -10-TAP	<i>sus1</i> -10-TAP in <i>SAC3</i> -GFP	This study
SAC3-GFP <i>sus1</i> -11-TAP	<i>sus1</i> -11-TAP in <i>SAC3</i> -GFP	This study
SAC3-GFP <i>sus1</i> -12-TAP	<i>sus1</i> -12-TAP in <i>SAC3</i> -GFP	This study
W303 a	<i>MATa, ade2-1, ura3-1, leu2-3, 112, his3-11, 15, trp1-1</i>	Thomas and Rothstein, 1989
W303 a <i>sus1</i> $\Delta$	<i>sus1</i> ::klURA3 in W303a	This study
<i>SUS1</i> -TAP	<i>SUS1</i> -TAP in W303a	This study
<i>sus1</i> -10-TAP	<i>sus1</i> -10 (E18A, S19A, G20A)-TAP in W303a	This study
<i>sus1</i> -11-TAP	<i>sus1</i> -11 (G37A, W38A)-TAP in W303a	This study
<i>sus1</i> -12-TAP	<i>sus1</i> -12 (V73A, D75A)-TAP in W303a	This study
<i>sus1</i> (E18A)-TAP	<i>sus1</i> (E18A)-TAP in W303a	This study
<i>sus1</i> (E18A/S19A)-TAP	<i>sus1</i> (E18A, S19A)-TAP in W303a	This study
<i>sus1</i> (S19A)-TAP	<i>sus1</i> (S19A)-TAP in W303a	This study
<i>sus1</i> (G20A)-TAP	<i>sus1</i> (G20A)-TAP in W303a	This study
<i>sus1</i> (G37A)-TAP	<i>sus1</i> (G37A)-TAP in W303a	This study

sus1 (W38A)-TAP	<i>sus1</i> (W38A)-TAP in W303a	This study
sus1 (W38D)-TAP	<i>sus1</i> (W38D)-TAP in W303a	This study
sus1 (V73A)-TAP	<i>sus1</i> (V73A)-TAP in W303a	This study
sus1 (V73D)-TAP	<i>sus1</i> (V73D)-TAP in W303a	This study
sus1 (D75A)-TAP	<i>sus1</i> (D75A)-TAP in W303a	This study
sus1 (D75G)-TAP	<i>sus1</i> (D75G)-TAP in W303a	This study
sus1 (D75P)-TAP	<i>sus1</i> (D75P)-TAP in W303a	This study
W303 $\alpha$	<i>MAT<math>\alpha</math></i> , <i>ade2-1</i> , <i>ura3-1</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-11</i> , <i>15</i> , <i>trp1-1</i>	Thomas and Rothstein, 1989
W303 $\alpha$ <i>sus1</i> $\Delta$	<i>sus1::klURA3</i> in W303 $\alpha$	This study
SUS1-FLAG	<i>SUS1</i> -FLAG in W303 $\alpha$	This study
sus1-10-FLAG	<i>sus1</i> -10 (E18A,S19A,G20A)-FLAG in W303 $\alpha$	This study
sus1-11-FLAG	<i>sus1</i> -11 (G37A, W38A)-FLAG in W303 $\alpha$	This study
sus1-12-FLAG	<i>sus1</i> -12 (V73A, D75A)-FLAG in W303 $\alpha$	This study
THP1-TAP	<i>MAT<math>\alpha</math></i> , <i>ade2-1</i> , <i>ura3-1</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-11</i> , <i>15</i> , <i>trp1-1</i> <i>THP1</i> -TAP::natNT2	Fischer et al., 2002
THP1-TAP <i>sus1</i> $\Delta$	<i>sus1::klURA3</i> in THP1-TAP	This study
THP1-TAP SUS1-FLAG	<i>SUS1</i> -FLAG in THP1-TAP	This study
THP1-TAP <i>sus1</i> -10-FLAG	<i>sus1</i> -10 (E18A, S19A, G20A)-FLAG in THP1-TAP	This study
THP1-TAP <i>sus1</i> -11-FLAG	<i>sus1</i> -11 (G37A, W38A)-FLAG in THP1-TAP	This study
THP1-TAP <i>sus1</i> -12-FLAG	<i>sus1</i> -12 (V73A, D75A)-FLAG in THP1-TAP	This study
<i>yra1</i> $\Delta$	<i>MAT<math>\alpha</math></i> , <i>ade2</i> , <i>leu2</i> , <i>trp1</i> , <i>ura3</i> , <i>yra1::HIS3</i> (pRS316- <i>YRA1</i> )	This study
<i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	<i>MAT<math>\alpha</math></i> , <i>ade2</i> , <i>leu2</i> , <i>trp1</i> , <i>ura3</i> , <i>sus1::kanMX4</i> , <i>yra1::HIS3</i> (pRS316- <i>YRA1</i> )	Rodriguez-Navarro et al., 2004
<i>yra1</i> $\Delta$ SUS1-TAP	<i>SUS1</i> -TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>yra1</i> $\Delta$ <i>sus1</i> -10-TAP	<i>sus1</i> -10 (E18A, S19A, G20A)-TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>yra1</i> $\Delta$ <i>sus1</i> -11-TAP	<i>sus1</i> -11 (G37A, W38A)-TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>yra1</i> $\Delta$ <i>sus1</i> -12-TAP	<i>sus1</i> -12 (V73A, D75A)-TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>yra1</i> $\Delta$ <i>sus1</i> (W38A)-TAP	<i>sus1</i> (W38A)-TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>yra1</i> $\Delta$ <i>sus1</i> (W38D)-TAP	<i>sus1</i> (W38D)-TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>yra1</i> $\Delta$ <i>sus1</i> (V73A)-TAP	<i>sus1</i> (V73A)-TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>yra1</i> $\Delta$ <i>sus1</i> (V73D)-TAP	<i>sus1</i> (V73D)-TAP in <i>sus1</i> $\Delta$ <i>yra1</i> $\Delta$	This study
<i>ada3</i> $\Delta$	<i>MAT<math>\alpha</math></i> , <i>his3</i> $\Delta$ 1, <i>15</i> , <i>leu2</i> $\Delta$ 0, <i>ura3</i> $\Delta$ 0, <i>lys2</i> $\Delta$ 0, <i>ada3::natNT2</i>	This study
<i>sus1</i> $\Delta$ <i>ada3</i> $\Delta$	<i>MAT<math>\alpha</math></i> , <i>his3</i> $\Delta$ 1, <i>15</i> , <i>leu2</i> $\Delta$ 0, <i>ura3</i> $\Delta$ 0, <i>lys2</i> $\Delta$ 0, <i>sus1::kanMX4</i> (pRS316- <i>SUS1</i> ), <i>ada3::natNT2</i>	generated by S. Lutz, Hurt lab

ada3Δ SUS1-TAP	<i>SUS1</i> -TAP in <i>sus1Δada3Δ</i>	This study
ada3Δ <i>sus1</i> -10-TAP	<i>sus1</i> -10-TAP (E18A, S19A, G20A) in <i>sus1Δada3Δ</i>	This study
ada3Δ <i>sus1</i> -11-TAP	<i>sus1</i> -11-TAP (G37A, W38A) in <i>sus1Δada3Δ</i>	This study
ada3Δ <i>sus1</i> -12-TAP	<i>sus1</i> -12-TAP (V73A, D75A) in <i>sus1Δada3Δ</i>	This study
ada3Δ <i>sus1</i> (W38A)-TAP	<i>sus1</i> (W38A) in <i>sus1Δada3Δ</i>	This study
ada3Δ <i>sus1</i> (W38D)-TAP	<i>sus1</i> (W38D) in <i>sus1Δada3Δ</i>	This study
ada3Δ <i>sus1</i> (V73A)-TAP	<i>sus1</i> (V73A) in <i>sus1Δada3Δ</i>	This study
ada3Δ <i>sus1</i> (V73D)-TAP	<i>sus1</i> (V73D) in <i>sus1Δada3Δ</i>	This study
YZS276	<i>MATa his3-11, -15 leu2-3, -112 trp1-1 ura3-1 ade2-1 hta-1htb-1::LEU2 hta-2htb-2 + pRS413-FLAG-HTB1-HTA1</i>	Robzyk et al., 2000
YZS276 <i>sus1Δ</i>	<i>MATa his3-11, -15 leu2-3, -112 trp1-1 ura3-1 ade2-1 hta-1htb-1::LEU2 hta-2htb-2 + pRS413-FLAG-HTB1-HTA1 sus1::natNT2</i>	generated by A. Köhler, Hurt lab

#### 4.2.4 Isolation of synthetic lethal mutants with the *sac3ΔCID* allele

Genetic interactions are termed synthetic lethal (SL) if two or more alleles that do not lead to cell death when appearing independently, become lethal when they are combined in one cell. A genetic SL screen as it is described here is a powerful tool to identify genes coding for proteins of a complementing pathway or even proteins interacting directly with the protein of interest.

The SL screen for *SAC3* had already been performed in the Hurt group by T. Fischer and A. Rácz as previously described (Appling, 1999; Guarente, 1993; Segref et al., 1997; Wimmer et al., 1992) and 50 SL mutants were available for further characterization.

*sac3Δ* SL mutants were selected for successful complementation of the strain by *SAC3* but not by a mutant of *SAC3* lacking the CID domain (*sac3ΔCID*). This was done by transforming all strains either with a plasmid containing *SAC3* (pRS315-*SAC3*) or a version of *SAC3* without the CID domain (pRS315-*sac3ΔCID*). Transformants on SDC-Leu were then restreaked to 5-FOA plates so that only cells could grow that were able to lose pHT4467-*SAC3*, carrying the *URA3* marker and a wild type copy of *SAC3*.

From the 50 *sac3Δ* SL mutant strains that analyzed, ten strains showed at least a significantly weaker complementation by *sac3ΔCID* than by *SAC3*.

The SL strains that were shown to require a functional *SAC3* for complementation were subsequently transformed with a yeast genomic library. This library contains genomic fragments of approximately 6 to 10kb inserted into an ARS/CEN vector pUN100 with a *LEU2* marker (Jansen et al., 1993). Once a library plasmid allowed for loss of the *URA3* plasmid carrying a wild type copy of *SAC3* and therefore growth on 5-FOA plates, the complementing plasmid was recovered, amplified and sequenced. Genes encoded by the recovered plasmid were cloned and tested individually for complementation of the SL strain.

### **4.3 Biochemical methods**

Protein separation by SDS gel electrophoresis and staining of protein gels with Coomassie Blue was performed as previously described (Sambrook and Russell, 2001).

#### **4.3.1 Whole yeast protein extract**

For the analysis of total proteins from yeast cells, a liquid culture of YPD was inoculated with yeast cells and grown overnight at 30°C in YPD or selective medium to an OD<sub>600</sub> between 0.5 and 1.0. A cell suspension equaling 5 OD<sub>600</sub> units was harvested in a centrifuge tube, washed with 1ml H<sub>2</sub>O and pelleted at 10000 rpm for 1 minute. After discarding the supernatant, the cells were lysed by vortexing for 3 minutes at RT in 400μl of 20% TCA (-20°C) and 200μl of glass beads. The precipitated protein was spun down for 5 minutes at 13000 rpm and 4°C. The supernatant was discarded and the pellet was washed with 1ml of 100% ethanol (-20°C). Following another centrifugation step at 13000 rpm and 4°C for 5 minutes, the supernatant was pipetted off and the pellet was dried in a Speed Vac (GMI Inc., Ramsey, USA) for 25 minutes. The dried pellet was resuspended in 150μl 1M Tris-

HCL (pH9.4) and 150µl 4xLaemmli sample buffer (Laemmli, 1970) and was then ready to use for gel electrophoresis and Western blotting.

#### **4.3.2 Tandem Affinity Purification of TAP fusion proteins**

Yeast cells from a 2l liquid culture of YPD were harvested at an OD<sub>600</sub> between 3.5-4.0 by centrifugation for 4 minutes at 4°C and 5000 rpm. After a washing step with 50ml 1xLB + 0.5mM PMSF and another round of centrifugation, the supernatant was discarded and the cell pellet was flash frozen in liquid nitrogen and stored at -20°C overnight.

On the second day, yeast pellets were thawed at 37°C and ~15ml of cold 1xLB + protease inhibitor mix FY (Serva, Heidelberg, Germany) + 1mM DTT was added to the cell suspension. Cell breakage was performed in a beater mill (Pulverisette, Fritsch, Idar-Oberstein, Germany) by glass bead lysis at 4°C (program: 500 rpm, 4 minutes, 1 minute break, 2 repetitions). The cell lysate was separated and the beads were additionally washed with 10ml 1xLB. Two centrifugation steps (4000 rpm, 10 minutes, 4°C and 19000 rpm, 45 minutes, 4°C) were applied to pellet crude yeast cell fragments. The supernatant of the lysate was then incubated with 300µl of equilibrated IgG-Sepharose<sup>TM</sup> 6 FastFlow beads for 1h at 4°C on a turning wheel. After this incubation, sepharose beads were washed with 15ml 1xLB + 0.5 mM DTT by gravity flow in 1ml Mobicol columns (MoBiTec, Göttingen, Germany). The column was plugged and the washed beads were resuspended in 300µl 1xLB + 0.5mM DTT + 20ng/µl TEV protease and incubated for 90 minutes at 16°C on a turning wheel to allow for selective cleavage of the TEV protease recognition site inside the TAP tag. The eluate from this TEV cleavage was eluted from the column by a centrifugal spin of 2000 rpm for 1 minute into a microcentrifuge tube and adjusted to 2mM CaCl<sub>2</sub> before the adjusted TEV eluate was incubated with equilibrated Calmodulin Sepharose 4B Resin (GE Healthcare, Chalfont St Giles, UK) for 1 hour at 4°C in a fresh Mobicol column. This resin was washed with 7.5ml 1xLB + 1mM DTT + 2mM CaCl<sub>2</sub> and finally, binding of the bait protein to the resin was disrupted by incubation of the beads with 600µl 10mM Tris-HCl (pH8) + 5mM EGTA (pH8) for 30 minutes at 37°C on a turning wheel. The purified protein solution

was eluted into a microcentrifuge tube by centrifugation at 2000 rpm for 1 minute at 4°C.

1xLB:

100mM NaCl

50mM Tris-HCl (pH7.5)

1.5mM MgCl<sub>2</sub>

0.15% NP-40

### **4.3.3 Trichloroacetic acid (TCA) protein precipitation**

This method to concentrate proteins by precipitation in aqueous solution was used to obtain a higher concentration of proteins purified by the TAP method. The solution was adjusted to 10% TCA to precipitate the protein and centrifuged for 15 minutes at 13000 rpm and 4°C to pellet the precipitate. After discarding the supernatant, the pellet is carefully washed by addition of 1ml of 100% acetone (-20°C) and another centrifugation step for 5 minutes at 13000 rpm and 4°C. The dried pellet was finally resuspended in 1x NuPAGE® Sample Buffer (Invitrogen, Carlsbad, USA) and analysed by SDS-PAGE on 4-12% gradient or 12% NuPAGE® Novex® Bis-Tris Gels (Invitrogen, Carlsbad, USA) run in MOPS or MES buffers (Good et al., 1966).

### **4.3.4 *In vitro* binding assays of recombinantly expressed proteins**

Recombinant proteins were expressed in *E. coli* BL21 codon plus RIL cells (Stratagene, La Jolla, USA). Cells were grown in minimal medium (MM; Sambrook and Russell, 2001) at 37°C to an OD<sub>600</sub> of 0.3 before expression was induced by addition of 0.5mM IPTG at 23°C for 3 hours.

Harvested cells were resuspended in 1xNB + 0.1% NP-40 + 1mM β-mercaptoethanol + protease inhibitor mix HP (Serva) and lysed using a Microfluidizer® (Microfluidics, Newton, USA). Lysates were then incubated with NiNTA agarose beads (Qiagen, Hilden, Germany) or with Glutathione Agarose 4B (Macherey-Nagel,

Düren, Germany) for 1 hour at 4°C on a turning wheel (for hexa-histidine tagged alleles of Sus1, GST-Sgf11 or co-expressed GST-SAC3<sup>573-805</sup> and Cdc31, respectively). After incubation, the NiNTA agarose was washed four times with 5ml of the lysis buffer with 30mM imidazole to reduce unspecific binding to the beads. GSH agarose was washed four times with 5ml of the lysis buffer. Proteins were eluted from NiNTA beads by incubation with 600µl of the lysis buffer with 150mM imidazole for 15 minutes at 4°C. The elution of the GST-SAC3<sup>573-805</sup>-Cdc31 heterodimer or GST-Sgf11 from glutathione beads was done in two cycles of incubation with 400µl lysis buffer containing 20mM glutathione and subsequent separation of eluate from the beads.

For the *in vitro* binding assays, purified Sus1 proteins and GST-Sgf11 or the SAC3<sup>573-805</sup>-Cdc31 heterodimer were combined at a 2:1 molar ratio and incubated with 300µl GSH beads for 30 minutes at 16°C in 1x binding buffer. The GSH agarose was washed as described above and eluted by incubation with 600µl lysis buffer containing 20mM glutathione. The protein eluates were concentrated by TCA precipitation and then analyzed by SDS-PAGE and Coomassie staining.

1xNB:

150mM NaCl

50mM CH<sub>3</sub>COOK

20mM Tris-HCl (pH7.5)

2mM (CH<sub>3</sub>COO)<sub>2</sub>Mg

1x binding buffer:

100mM NaCl

50mM HEPES (pH7.5)

0.5mM DTT

#### 4.3.5 Analysis of changes in global ubiquitin levels

Immunoprecipitation of FLAG-tagged histone H2B in *sus1Δ*, *SUS1*-TAP or mutant *sus1*-TAP backgrounds was performed as previously described (Kao and Osley, 2003). Proteins from yeast cells purified by the FLAG tag were separated on SDS-PAGE and FLAG-tagged proteins (unmodified and ubiquitinated FLAG-H2B) were further analyzed by Western blotting and detection by an α-FLAG antibody.



## 4.4 Miscellaneous

### 4.4.1 Western blotting

Proteins were transferred from SDS-PAGE to a PROTRAN<sup>TM</sup> nitrocellulose membrane (GE Healthcare, Chalfont St Giles, UK) by electric current as previously described (Sambrook and Russell, 2001). After blotting the proteins to the membrane for 45 minutes at 11V using a TransBlotSD semi-dry blotting apparatus (BIO-RAD, Munich, Germany), the membrane was stained with Ponceau Red (Serva, Heidelberg, Germany) and rinsed with H<sub>2</sub>O to reversibly stain and visualize blotted proteins. For size comparison, I ran a PageRuler<sup>TM</sup> Prestained Protein Ladder (Fermentas, Burlington, Canada) with every protein gel. The immobilized proteins were detected by an indirect enzyme-linked immunological method. Unspecific binding of the antibody to the membrane was reduced by incubating the membrane for 1 hour at RT or overnight rocking at 4°C in blocking buffer (1xPBS + 0.1% Tween-20 + 5% milk powder). After blocking, the membrane was incubated with a primary antibody in blocking buffer rocking for 1 hour at RT or overnight at 4°C. Three washing steps of 12 minutes each at RT with 1xPBS + 0.1% Tween-20 were performed to wash away unspecifically bound antibody. If a secondary antibody was necessary for detection, this antibody which was conjugated with horseradish peroxidase, was again added in blocking buffer and the membrane was incubated rocking for 1 hour at RT or overnight at 4°C. Excess antibody was washed off by washing the membrane as described above and detection of HRP-conjugated antibodies bound to proteins on the membrane was performed using the ECL chemiluminescence system (GE Healthcare, Chalfont St Giles, UK). Documentation of luminescent signals was done by exposition to Amersham Hyperfilm ECL films (GE Healthcare, Chalfont St Giles, UK) for 5 seconds to 30 minutes.

Table IV: **Antibodies used in this study**

Primary antibody (with dilutions)	Source	Secondary antibody (with dilutions)	Source
$\alpha$ -protein A (1:3000)	DAKO, Hamburg, Germany	no secondary antibody necessary	
$\alpha$ -Sac3 (1:2000)	R. Kölling, U. of Hohenheim, Germany	goat $\alpha$ -rabbit (1:8000)	Bio-Rad, Munich, Germany
$\alpha$ -Cdc31 (1:300)	E. Schiebel, ZMBH, Heidelberg, Germany	mouse $\alpha$ -goat (1:3000)	dianova, Hamburg, Germany
$\alpha$ -CBP (1:10000)	BioCat, Heidelberg, Germany	goat $\alpha$ -rabbit (1:8000)	Bio-Rad, Munich, Germany
$\alpha$ -Arc1 (1:5000)	Hurt laboratory	goat $\alpha$ -rabbit (1:8000)	Bio-Rad, Munich, Germany
$\alpha$ -FLAG (1:1000)	Sigma-Aldrich, St. Louis, USA	goat $\alpha$ -mouse (1:3000)	Bio-Rad, Munich, Germany

#### 4.4.2 Fluorescence microscopy

Fluorescence microscopy was performed using an Imager Z1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 100x/63x NA 1.4 Plan-Apo-Chromat oil immersion lens (Carl Zeiss, Oberkochen, Germany) and using HE-eGFP (for GFP microscopy), HE-DsRed (for Cy3-oligo-dT microscopy), DICIII (for Nomarski images) or DAPI filters. Images were acquired with an AxioCam MRm camera and AxioVision 4.3 software (Carl Zeiss, Oberkochen, Germany).

##### 4.4.2.1 GFP microscopy

For the analysis of the localization of proteins, yeast strains expressing fusion proteins with GFP (see table III) were grown to mid-logarithmic phase ( $OD_{600}$  0.3-0.5) in SDC-leu liquid medium. Cells were harvested by centrifugation, washed with  $H_2O$  and applied onto glass slides (ROTH, Karlsruhe, Germany). Localization of the fluorescent proteins was documented as described above (exposition time for Nomarski filter: 20 ms, HE-eGFP filter: 1400 ms).

#### 4.4.2.2 *In situ* hybridization of *poly(A)<sup>+</sup>* RNA

On the first day, yeast strains expressing wild-type or mutant Sus1 were grown at 30°C in YPD to an OD<sub>600</sub> of 0.3 before shifting to 37°C for 2 hours. A first fixation step was performed by adding 37% formaldehyde to a final concentration of 4% formaldehyde and incubation at 37°C for 15 minutes. Cells were harvested and further fixated by resuspending in 4% formaldehyde + 0.1M KP<sub>i</sub> (pH6.4, potassium phosphate buffer) and incubating for 1.5 hours at RT on a turning wheel. Following two rounds of washing in 0.1M KP<sub>i</sub> pH6.4, the cells were washed again in 1ml washing buffer (0.1M KP<sub>i</sub> + 1.2M sorbitol) and then resuspended in 1ml washing buffer + 500µg of 100T zymolyase (Seikagaku, Tokyo, Japan). The enzymatic digest of the cell wall took place for 30 minutes at 30°C on a turning wheel. The spheroplasts are centrifuged for 3 minutes at RT and 3300 rpm, washed with wash buffer and resuspended in 2 vol. of wash buffer. 20µl of the cell suspension was pipetted onto 5-well glass slides (Thermo Fisher Scientific, Waltham, USA) coated with poly-L-lysine and incubated for 5 minutes at RT. Excess cell suspension was removed by aspiration and non-adhering cells were further washed away by addition of 100µl of 2xSSC and removal by aspiration. After another round of addition of 100µl of 2xSSC and subsequent removal, each well was covered in 12µl of prehybridization buffer and incubated for 1 hour at 37°C in a humid chamber. 1µl of 1µM 5' Cy3-labelled oligo-dT per well was added to the prehybridization mix and incubated overnight at 37°C in a dark humid chamber.

On the second day, the glass slide carrying the hybridized spheroplasts was stringently washed by rocking in 2xSSC for 5 minutes. Another washing step was done in 1xSSC before the slide was incubated in 1xSSC + 2µl of 2.5mg/ml DAPI (4', 6-diamidino-2-phenylindole; Merck, Darmstadt, Germany) for 3 minutes rocking at RT. Subsequently, the glass slide was washed with 1xSSC and finally with 0.5xSSC. All these steps were done in a staining jar (ROTH, Karlsruhe, Germany) in the dark. The slide was dried for ~60 minutes at RT, wells were coated with 10µl Mowiol solution and a cover slip (ROTH, Karlsruhe, Germany) was mounted onto the glass slide. The Mowiol was hardened at RT and spheroplasts were analyzed under a fluorescence microscope (see above). Images were acquired for (exposition time for DAPI filter: 60 ms, HE-DsRed filter: 500 ms).

1xSSC:

150mM NaCl  
15mM trisodium citrate (Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>)

prehybridization buffer:

50% formamide  
10% dextrane sulphate  
125µg/ml E. coli tRNA (Sigma-Aldrich, St. Louis, USA)  
500µg herring sperm DNA (Sigma-Aldrich, St. Louis, USA)  
4x SSC  
0.02% polyvinyl pyrrolidone  
0.02% Ficoll-400 (GE Healthcare, Chalfont St Giles, UK)  
1mM DTT

Mowiol solution

6g glycerol and 2.4g Mowiol (Calbiochem, San Diego, USA) were mixed, 6ml H<sub>2</sub>O added and the mix stirred for 2 hours at RT. After this time, the solution was stirred with 12ml 0.2M Tris-HCl (pH8.5) for 1 hour at 50°C. When the solution cleared, it was centrifuged for 15 minutes at 5000rpm and the supernatant aliquotted and stored at -20°C.

**4.4.3 *in silico* analysis**

Secondary structure prediction of the sequence of Sus1 was done using the program PsiPred (<http://bioinf.cs.ucl.ac.uk/psipred/>). Sequences of Sus1 from selected species including protozoa, fungi, plants and metazoan were aligned with ClustalW2 ([www.ebi.ac.uk/clustalw2/](http://www.ebi.ac.uk/clustalw2/)) and shaded using JalView (Waterhouse et al., 2009). Images showing details from the crystal structure of the Sac3 (723-805):Cdc31:Sus1 complex (Jani et al., 2009; protein data bank accession number 3FWC) were generated with PyMOL (<http://pymol.sourceforge.net/>).

## 5. ABBREVIATIONS

<b>5-FOA</b>	5' fluoroorotic acid
<b>CBP</b>	calmodulin binding protein
<b>CD</b>	circular dichroism
<b>CID</b>	Cdc31-interacting domain
<b>Da</b>	Dalton
<b>DAPI</b>	4,6-diamidino-2-phenylindole
<b>DNA</b>	deoxyribonucleic acid
<b>DTT</b>	dithiothreitol
<b>DUB module</b>	deubiquitinating module
<b>eIF5A</b>	eukaryotic translation initiation factor 5A
<b>EJC</b>	exon-junction complex
<b>FG repeats</b>	phenylalanine-glycine repeats
<b>FRET</b>	fluorescence resonance energy transfer
<b>GDP</b>	guanosine diphosphate
<b>GFP</b>	green fluorescent protein
<b>GSH</b>	glutathione
<b>GTP</b>	guanosine triphosphate
<b>HCl</b>	hydrochloric acid
<b>HRP</b>	horseradish peroxidase
<b>IPTG</b>	Isopropyl $\beta$ -D-1-thiogalactopyranoside
<b>LB medium</b>	lysogeny broth medium
<b>LRR</b>	leucine-rich repeat
<b>MES</b>	2-(N-morpholino) ethanesulfonic acid
<b>MOPS</b>	3-(N-morpholino) propanesulfonic acid
<b>mRNA</b>	messenger RNA
<b>mRNP</b>	messenger ribonucleoprotein particle
<b>NES</b>	nuclear export signal
<b>NLS</b>	nuclear localization signal
<b>NPC</b>	nuclear pore complex
<b>OD</b>	optical density
<b>ORF</b>	open reading frame

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<b>pA</b>	protein A
<b>PBS</b>	phosphate buffered saline
<b>PCR</b>	polymerase chain reaction
<b>PMSF</b>	phenylmethanesulphonyl fluoride
<b>RBD</b>	RNA-binding domain
<b>RIL</b>	revolutions per minute
<b>RNA</b>	ribonucleic acid
<b>rpm</b>	revolutions per minute
<b>rRNA</b>	ribosomal RNA
<b>RT</b>	room temperature
<b>SAGA</b>	Spt-Ada-Gcn5 acetyltransferase
<b>SDC</b>	synthetic defined complete (medium)
<b>SDS-PAGE</b>	sodium dodecylsulfate polyacrylamide gel electrophoresis
<b>SE</b>	synthetic enhancement / synthetically enhanced
<b>SL</b>	synthetic lethality / synthetically lethal
<b>SNARE</b>	soluble N-ethylmaleimide-sensitive-factor attachment receptor
<b>SSC</b>	saline sodium citrate
<b>TAP</b>	tandem affinity purification
<b>TBP</b>	TATA-binding protein
<b>TCA</b>	trichloroacetic acid
<b>TEV</b>	tobacco etch virus
<b>TREX</b>	transcription and mRNA export complex
<b>tRNA</b>	transfer RNA
<b>ts</b>	temperature-sensitive
<b>UBA</b>	ubiquitin-associated
<b>UTR</b>	untranslated region
<b>WCE</b>	whole cell extract
<b>WT</b>	wild-type
<b>YPD</b>	yeast peptone dextrose

## **6. OWN PUBLICATION ON THE TOPIC**

Klößner, C., Schneider M., Lutz S., Jani D., Kressler D., Stewart M., Hurt E. and Köhler A. (2009). Mutational Uncoupling of the Role of Sus1 in Nuclear Pore Complex Targeting of an mRNA Export Complex and Histone H2B Deubiquitination. *J Biol Chem*, 284(18):12049-56

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